HEPATIC FIRST-PASS CLEARANCE OF ORAL GALACTOSE AND ITS POTENTIAL USE IN THE ASSESSMENT OF PORTASYSTEMIC SHUNTING IN PORTAL HYPERTENSION

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DECLARATION

The studies contained within this thesis were performed between November 1986 and November 1989 when I was a Research Assistant in the University Department of Surgery, Glasgow Royal Infirmary.

I declare that I am the sole author of this thesis. The investigations and data collection were carried out and processed by myself. Where assistance has been obtained from others, such help has been freely acknowledged.

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SUMMARY

Portasystemic shunting is an important consequence of all diseases that lead to portal hypertension. The clinical-pathological problems associated with portasystemic shunting, such as hepatic encephalopathy and oesophageal varices, remain a potent cause of much morbidity and mortality. The aetiology of hepatic encephalopathy remains poorly understood, but it is thought that the more subtle sequallae of portasystemic shunting, such as hormonal (Fischer et al, 1974), and nutritional (Phear et al, 1955; Fischer and Baldessarini, 1971) changes contribute in varying degrees to the onset and progression of the condition. Quantitation of the magnitude of portasystemic shunting is thus potentially most useful in the study of hepatic encephalopathy.

Up to now it has not been practical to measure on a routine basis the fraction of portal blood that bypasses functioning hepatocytes to reach the systemic circulation. The available methods are either highly invasive (Okuda et al, 1977), time consuming (McLean et al, 1979), or suffer from known measurement inaccuracies (Porchet and Bircher, 1982; Cavanna et al, 1987), and their applications have been limited to a few studies. The purpose of this thesis is to describe and assess a novel, non-invasive procedure that

approaches quantitative assessement of portasystemic shunting.

The principle of the method consists of a comparison of the systemic availability of an oral and intravenous dose of galactose. The ability of galactose to quantitate the magnitude of portasystemic shunting associated with portal hypertension has been examined in patients with documented oesophageal varices and in a rat model of prehepatic portal hypertension using partial portal vein ligation. The rat model had been established and characterised previously in the University Department of Surgery, Glasgow Royal Infirmary (Geraghty et al, 1989).

Data from the studies described in this thesis has shown a marked difference in the systemic availability of galactose between control patients and patients with liver disease and/or portal hypertension. In the former group only 14 percent of the oral dose appeared in the systemic circulation, whereas in portal hypertensive patients an average of 72 percent (range 38% to 108%) was detected systemically. The absence of a gold standard for portasystemic shunting measurements, and ambiguity in the definition of portasystemic shunting itself, preclude direct validation of the measurements in man. However, the key assumptions about galactose elimination kinetics underlying the measurement technique were tested independently and found to be

satisfied.

The initial application of the technique in the rat model of prehepatic portal hypertension, in which independent measurements of portasystemic shunting could be performed using a radioactive microsphere technique, failed to show a difference in the systemic availability of oral galactose between control and portal hypertensive rats. Further studies showed this was largely due to erratic and incomplete absorption of galactose from the gastrointestinal tract.

A more comprehensive investigation was carried out and the kinetics of hepatic galactose elimination in the rat was studied by direct infusion of galactose into the portal vein. From this it was shown that, in contrast to man, hepatic extraction efficiency in this animal model had a high dependence on circulating blood galactose concentrations. This compromised the accuracy of the technique for quantitation of portasystemic shunting in individual animals because of the variability in kinetic parameters. However, there was a highly significant correlation between the systemic availability of intraportally administered galactose in rats and portasystemic shunting as measured with radioactive microspheres.

The galactose technique described in this thesis may thus be regarded as a tool to assess the magitude of portasystemic shunting, and may have a role in the

study of the associated complications of portal hypertension and liver disease.

CHAPTER ONE HISTORICAL REVIEW

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INTRODUCTION

Portal hypertension is described as an elevation of pressure in the portal venous system, and is secondary to various disease processes which affect the liver and/or the portal vein. The elevation of pressure in the portal venous system precipitates the opening of venous channels which carry portal venous blood away from the liver directly into the systemic circulation. The development of portasystemic shunts, as they are known, are responsible for the eventual clinical manifestations of portal hypertension, as well as various other problems. Oesophageal vaices and hepatic encephalopathy are by far the most clinically significant complications of portasystemic shunting and both are a potent cause of much morbidity and mortality. Various other problems associated with portasystemic shunting are septicemia, hypergammaglobulinemia, and drug toxicity.

Oesophageal varices are one of the many channels by which portal venous blood can be diverted into the systemic circulation and their rupture has been linked to the height of pressure in the portal venous system (Orloff et al, 1963). The more subtle sequellae of portasystemic shunting such as the diversion of gut-derived compounds, for example ammonia (Bollman, 1961), and gastrointestinal hormones (Fischer et al, 1974)

into the systemic circulation are thought to explain, at least in part, the onset and development of hepatic encephalopathy. The aetiology of the condition, however, remains poorly understood. The possibility of gaining a fuller understanding into the onset and development of hepatic encephalopathy may be realised if a means of quantitating the magnitude of portasystemic shunting could be developed. Up to now it has not been practical on a routine basis to measure the amount of portal blood that bypasses functioning hepatocytes to enter the systemic circulation. Methods currently available are either highly invasive (Okuda et al, 1977) and time consuming (McLean et al, 1979). The aim of this thesis is to develop and assess a novel, non-invasive method by which the degree of diversion of portal blood into the systemic circulation can be quantitated. The method, described in a subsequent chapter, is assessed in control subjects and patients with portal hypertension, and in a rat model of prehepatic portal hypertension.

This review of the literature is aimed at describing the development of portasystemic shunting, discussing the consequences of a developed collateral circulation and its contribution to the maintenance of portal hypertension, and describing the attempts that have been made at quantitating the magnitude of portasystemic shunting.

The review is divided into five sections. The first two sections describe the circulation of blood in the normal liver and portal vein and detail the morphological changes that occur to both the portal vein and the liver with the development of portal hypertension. This is linked to the development of a collateral circulation.

The third section discusses the pathophysiology of portal hypertension emphasising that the maintenance of a chronically elevated portal venous pressure in the face of a developed collateral circulation may be the result of an increased splanchnic inflow. This increase in splanchnic blood flow appears to be linked to the diversion of vasoactive substances derived from the gastrointestinal tract and normally metabolised by the liver through portasystemic shunts.

The consequences of portasystemic shunting are described in the fourth section of this review and emphasis is placed on the development of hepatic encephalopathy through nutritional and hormonal abberations. Also stressed is the fact that the potential clinical usefulness of quantitating portasystemic shunting is in the study of hepatic encephalopathy.

The final section of the historical review describes methods which have been used to quantitate the magnitude of portasystemic shunting. The relative

merits and drawbacks of these techniques are discussed and emphasised.

PORTAL AND LIVER CIRCULATION

The dual supply of blood to the liver is provided through the portal vein and the hepatic artery. The portal vein itself is composed of four large veins which collect the venous blood from the viscera of digestion i.e. the stomach, intestine and pancreas, and from the spleen. The hepatic artery in turn carries blood directly to the liver from the heart. Both vessels seperately enter the liver through the porta hepatis, wherein the portal vein and the hepatic artery divide into branches to the right and left lobes of the liver (Rappaport, 1976). Both the portal vein and the hepatic artery branch extensively until both the high pressure of the hepatic arterial stream (120mmHg) and the low pressure portal venous stream (9mmHg) are united at the inlets of the hepatic sinusoids, which are thought to be controlled by inlet sphincters (Rappaport, 1976). In addition to these principal blood flow conduits, there are presinusoidal intrahepatic connections between the hepatic arterial and portal venous branches, though which it is estimated that 30% of the hepatic arterial blood is shunted into the portal venous system before reaching the sinusoids (Rabinovici and Vardi, 1965). At this level the circulation to the hepatic acinus (a microvascular unit of the liver) consists of terminal branches of the

hepatic arteriole and portal venule. A cluster of parenchymal cells are grouped around this microcirculation, together with terminal branches of the hepatic venule, which eventually unite to form the hepatic vein and so return blood to the vena cava (Rappaport, 1973).

The liver receives 25 percent of the cardiac output. Of the total hepatic blood flow 20 to 33 percent is supplied by the hepatic vein, the remaining two-thirds of the hepatic blood supply is portal venous blood (Lautt and Greenway, 1987). Liver blood flow measurements based upon the rate of disappearance of colloidal particles from peripheral blood, and bromsuphalein, indocyanine green, and galactose clearance have shown hepatic blood flow to range from 1,200 to 1,663 ml/min (Keiding, 1988; Caesar et al, 1961; Shaldon et al, 1961; Bradley et al, 1945). With the advent of more modern techniques, such as pulsed doppler flowmetry, it is now possible to continuously measure blood flows in the portal vein and the hepatic artery (Braillon and Brody, 1988).

Studies carried out in animals have suggested that blood flow in the portal vein may be streamlined (Copher and Dick, 1928). Three distinct and seperate currents have been identified in dogs arising from three sources, namely, the splenic vein, the superior mesenteric vein and its branches and the inferior

mesenteric vein. More recent work in patients has produced similar findings (Sherlock, 1978), although variability of the streamlines in the portal vein appear to be commonly observed.

In comparison to the extrahepatic studies on the vessels supplying the liver, intrahepatic blood flow distribution studies are comparatively sparse. However, studies carried out in which microspheres were injected into either the portal vein or hepatic artery (Greenway and Stark, 1971) suggest that there are no gross differences between the lobes of the liver in the proportion of either arterial or portal venous blood received. Futher, elevation in venous pressure, reduction of portal venous flow and stimulation of hepatic nerves or noradrenaline infusion do not result in flow redistribution within the liver (Gumucio, 1983; Greenway et al, 1972; Cousineau et al, 1985).

As flow in any vessel is a function of both pressure and resistance to flow, then liver blood flow can be influenced by hepatic arterial vascular resistance, the vascular resistance of the intestine, which governs the inflow of blood into the portal vein and intrahepatic portal vascular resistance.

Portal blood flow is exclusively determined by events in the splenic and gastrointestinal beds and not by events occuring in the liver (Greenway and Stark, 1971). Indeed the resistance that the splanchnic organs

offer to blood flow by far exceeds that offered by the liver, hence the resistance by the splanchnic organs to blood entering the portal vein has the dominant influence on portal blood flow. The only control of blood flow within the liver is via the hepatic artery. The factors which influence hepatic arterial resistance are thought to be both intrinsic and extrinsic in nature. However, as yet these factors have not been completely clarified (Greenway and Stark, 1971; Lautt and Greenway, 1987).

The inter-relationship of both the portal vein and the hepatic artery further serves to regulate blood flow. Ternberg and Butcher (1965) measured hepatic arterial and portal venous blood flows in the dog using electromagnetic flow meters, and demonstrated that a decrease in portal venous blood flow was associated with an increase in the hepatic arterial blood flow. The reciprocal relationship between portal and hepatic arterial flow is not only maintained but is enhanced in portal hypertension (Baker et al, 1974; Zimmon and Kessler, 1980). The mechanism behind the reciprocity however, is not yet understood, but it is thought that the hepatic artery may serve as a buffer for flow changes in the portal vein in a manner that tends to maintain a haemostatic environment within the liver, however, the hepatic artery cannot compemsate quantitatively for altered portal flow (Greenway and

Stark, 1971).

PORTAL VEIN AND LIVER MORPHOLOGY IN PORTAL HYPERTENSION

The fundamental mechanism underlying the pathophysiology of portal hypertension has been widely accepted for numerous years as an increased resistance to portal venous flow (Herrick, 1907). The rise in portal venous pressure is secondary to the morphological alterations occuring within the liver and the portal vein under varying disease conditions. The most frequently observed conditions precipitating portal hypertension are cirrhosis, alcoholic hepatitis, liver carcinomas, and parasitic invasion of the liver (Reynolds, 1969). Extrahepatic portal hypertension occurs if the portal or splenic veins are partly or completely occluded (Reynolds, 1969). Hepatic cirrhosis is primarily the cause of portal hypertension in the western world, but extrahepatic obstruction of the portal vein also produces equivalent pressure increases (Rousselot et al, 1959).

McIndoe (1928) and Epplen (1922) in examining vascular lesions in liver cirrhosis noted obvious superficial alterations in the liver. A marked diminution in liver size was frequently observed. However, on occasions the converse occurs, and the liver is observed to undergo an increase in size (Herrick, 1907). Further the liver appeared hobnailed, and stiff with an increased consistancy.

Intrahepatic vascular transformations occuring within a cirrhotic liver were examined by use of celloidin casts of the portal venous pathways. From such casts, McIndoe (1928) and Popper et al (1952) were able to demonstrate in patients and rats (Popper et al, 1952) a marked reduction in the total vascular bed together with a stenosis and attenuation of the main portal trunks and numerous portahepatic anastomoses (intrahepatic shunts). Portograms performed by Rousselot et al (1959) on patients and cirrhotic rats showed clearly the portal circulatory derangement observed in cirrhosis of the liver, including the development of an extrahepatic collateral circulation.

Histological and serial sectioning of the liver have provided an explanation for the altered vascular pattern in the liver together with the liver's hobnailed appearance. Comprehensive reviews of the liver by Rappaport (1976) and Sherlock (1981) detail the necrosis of the parenchymal cells, the collapse of hepatic lobules, formation of diffuse septa, and the nodular regrowth of liver cells. It is the regeneration of these nodular liver cells, which explains the hobnailed appearance of the liver together with the distortion of the hepatic vascular tree.

In contrast, portal hypertension resulting from extrahepatic occlusion of the portal vein does not result in notable morphological changes in the liver

(Simonds, 1936), indeed complete occulsion of the portal vein as performed in the monkey by Child and associates (1950) failed to demonstrate at hepatic biopsy any evidence of morphological change. The portal vein itself, however, can undergo various degrees of occlusion which give rise to a range of transformations in the appearance of the portal vein (Philips, 1988). Turner et al (1957) showed by use of venograms and autopsy studies on patients a dramatic transformation of the portal vein into a cavernous mass of small tortuous vessels. By contrast, Simonds (1936) and Whipple (1945) showed from hepatic autopsy studies the more subtle changes of the portal vein, which involve replacement of the vein or its main tributaries with fibrous tissue or scar tissue with little or no canalization. However, whichever morphological form the portal vein assumes, the resultant increase in portal venous pressure inevitably precipitates the formation of extrahepatic shunts (Rousselot et al, 1959).

THE PATHOPHYSIOLOGY OF PORTAL HYPERTENSION

In an attempt to understand the fundamental cause of the chronic increase in portal pressure two opposing theories have developed. The first, and the most obvious theory, is the "obstruction" or "backward flow" theory, which was first proposed by Whipple (1945) and was based upon the suggestion by Herrick (1907) that intrahepatic fibrosis was the precipitating factor in the elevation of portal venous pressure. The opposing theory, the "forward flow" theory, was suggested initially by Banti's comments on his patients with congestive splenomegaly in 1894 (Banti, 1894).

1. "Backward flow" theory

The "backward flow" theory of portal hypertension is based upon the concept of a raised portal venous pressure secondary to an increased resistance to portal venous flow. The term does not necessarily imply a reversal in direction of blood flow.

Supportive evidence for the "backward flow" theory has arisen from abundant clinical and pathological data (Popper et al, 1952) together with evidence of a decreased hepatic sinusoidal and venular drainage capacity (Friedman and Weiner, 1951; Ketly et al, 1950).

Prehepatic portal hypertension secondary to portal

vein obstruction has also been shown to increase resistance in the portal venous system (Halvorsen and Myking, 1979), and increased rat stomach weights in the face of obstructed venous outflow (Baronofsky et al, 1945), together with dilation of the gastric veins (Rousselot et al, 1959) has been cited as evidence of the stasis in the circulation in portally hypertensive splanchnic organs. Such observations have led to a frequent analogy being made between "congestive splanchnic failure" in portal hypertension and "congestive cardiac failure". A reduced hepatic blood flow in patients with cirrhosis of the liver (Bradley et al, 1952) has also been considered indicative of a reduced splanchnic inflow, and measurements of a reduced blood flow in the portal vein by means of electromagnetic flowmeters (Ferguson, 1963; Moreno et al, 1967) would appear to substantiate hepatic blood flow findings. However, since neither total liver nor portal venous blood flow necessarily includes flow in the portasystemic shunts, these direct flow measurements may not reflect accurately the level of splanchnic inflow in portal hypertension. Despite this, a wealth of evidence exists which appears to corroborate the backward flow theory, thus it has been generally accepted for numerous years, that a reduced splanchnic circulatory flow exists in both intrahepatic and extrahepatic portal hypertension.

2. "Forward flow" theory

(a) Systemic haemodynamics in portal hypertension

Evidence of an alternative hypothesis underlying the pathophysiology of portal hypertension was first provided by Kowalski and Abelmann in 1953.

The existence of a hyperkinetic state was found indirectly by both investigators when they demonstrated an increase in resting cardiac output in one third of patients suffering with hepatic cirrhosis. This observation was later confirmed by investigators Murray et al (1958), who further suggested that the elevated cardiac output was one of the factors responsible for the frequent occurence of warm extremities and peripheral pulsations observed in patients with hepatic cirrhosis. This elevated cardiac output, or hyperkinetic haemodynamic state in cirrhotic patients was found to closely resemble the "shunt circulation" occuring in patients with arterio-venous (A-V) fistulae (Johnson et al, 1969) and further reports of arteriovenous fistulae in the spleen, stomach and lower oesophagus (Peters and Womack, 1961) and lung (Rydell and Hoffbauer, 1956) of patients with hepatic cirrhosis has led to the suggestion that the elevated cardiac output is secondary to the development of A-V fistulae. In conjunction with the raised cardiac output, Kowalski and Abelmann (1953) and Murray et al (1958) were also

able to demonstrate a reduction in peripheral systemic vascular resistance in patients with elevated cardiac output, and attributed this fall to the increased presence of a "vasodilator material" normally metabolised by the liver (Shorr et al, 1951). This finding has also been supported by the failure to demonstrate an increased cardiac output in patients with extrahepatic portal hypertension and normal liver function (Murray et al, 1958). Subsequent studies have focused on organ blood flow in cirrhosis and work carried out by Kontos et al (1964) demonstrated an increased blood flow to the upper limb and a reduced arteriovenous oxygen difference.

(b) Splanchnic haemodynamics in portal hypertension

It is generally accepted that increased resistance to flow in the portal vein and liver will raise portal pressure (Halvorsen and Myking, 1979; Popper et al, 1952), but the maintenance of such an elevated portal pressure in the presence of extensive collateral communications between the portal venous system and the systemic circulation could not by means of the "backward flow" theory be rationally explained. Over the last 20 years various investigators have proposed that portal hypertension is maintained after the

development of portasystemic shunts as a result of an increased splanchnic inflow.

Indirect evidence of an elevated splanchnic blood flow was provided by Williams et al (1968) and later by Gitlin et al (1970) and Witte et al (1972). All three investigators showed an increase in total splenic blood flow in cirrhotic patients as compared to controls, and suggested that an elevated splenic blood flow would contribute to the elevation in portal venous pressure. Demonstration that portal blood is highly oxygenated (Womack and Peters, 1961; Witte et al, 1969) a feature which is in direct contrast to the stagnant circulation of congestive heart failure, often used as a comparative description to that of the splanchnic circulation, is also suggestive of an elevated splanchnic inflow.

The first direct evidence of an elevated splanchnic blood flow was, however provided by Kotelanski et al (1972), who demonstrated that the mean transit time of radiolabelled albumin from the superior mesenteric and hepatic arteries to the hepatic veins was significantly reduced in patients with hepatic cirrhosis. Since portal venous pressure is the result of the interplay between portal venous blood flow and the vascular resistance offered to that flow, then such evidence from Kotelanski et al (1972) and Williams et al (1968) has suggested that increases in splanchnic blood flow

may be an important factor in the pathogenesis of portal hypertension. Subsequent studies were, therefore aimed at splanchnic blood flow and its relationship to portal pressure. Witte et al (1974) measured, in dogs, superior mesenteric artery flow and portal venous pressure following intravenous infusion of glucagon. Glucagon simulated a hyperdynamic splanchnic blood flow causing a two fold increase in flow in the superior mesenteric artery without any rise in portal venous pressure. In contrast, constriction of the portal vein produced an increase in pressure, and in seperate animals both glucagon infusion and portal vein constriction produced a much greater rise. Thus it was proposed that a combined effect of elevated splanchnic inflow and resistance to portal venous flow was responsible for the level of portal pressure and the production of active congestion. Later work by Witte et al (1976) substantiated earlier findings and demonstrated that the sequential rise in portal venous pressure secondary to increasing constriction of the portal vein is enhanced during increasing arterial infusions of glucagon. Thus, together with the finding that blood volume was increased in patients with cirrhosis (Zimmon and Kessler, 1974), subsequent studies have focused on the role of elevated splanchnic inflow rather than increased resistance to portal venous flow in the pathogenesis of portal hypertension

(Vorobioff et al, 1983; Vorobioff et al, 1984). Subsequent studies in rat models of cirrhosis and chronic extrahepatic portal hypertension have shown conclusively that portal venous inflow is increased in these models although there remains a conflict of evidence about whether increased downstream resistance also contributes to elevated portal venous pressure (Blanchet and Lebrec, 1982; Benoit et al, 1985; Sikuler et al, 1985).

As discussed earlier, the development of a hyperdynamic systemic circulation has been attributed to the presence of a "vasodilator material" normally metabolised by the liver (Shorr et al, 1951). The same "vasodilator material" is also thought to be responsible for the increase in portal venous inflow. Theories about the mediator of the increased portal venous inflow proposed by the "forward flow" theory abound and at present the gastrointestinal hormone glucagon seems the most likely candidate as the mediator of this process (Sherwin et al, 1974; Sikuler et al, 1984; Benoit et al, 1984). Glucagon is known to induce a significant reduction of mean arterial pressure, total vascular resistance and splanchnic resistance and an increase intestinal and arterial hepatic blood flow (Feruglio et al, 1966; Farah, 1983). Glucagon concentrations are elevated in cirrhotic patients particularly in association with portasystemic

shunting (Sherwin et al, 1974) mainly as a consequence of pancreatic hypersecretion (Sherwin et al, 1978). However, correlation to hepatocellular injury has also been reported (Smith-Laing et al, 1980).

Benoit et al (1984) have found increased concentrations of glucagon in portal vein constricted rats and intra-arterial infusions of glucagon produced a 20% reduction of intestinal vascular resistance. Sikuler et al (1984) have observed similar increased concentrations of glucagon in portal vein ligated rats, however no correlation was found between the hyperdynamic circulation and glucagon blood levels.

While the initial mechanism of increased portal pressure may be related to a distorted intrahepatic vascular bed with subsequent increased resistance, the development of a collateral circulation acts to reduce resistance to portal blood flow. It is at this stage that the increased portal venous inflow might play an important role in the maintenance of portal hypertension. Thus the development of portasystemic shunting itself may contribute, by diverting humoral vasodilator factors released from the gastrointestinal tract into the systemic circulation, to the maintenance of portal hypertension.

CONSEQUENCES OF PORTASYSTEMIC SHUNTING

Both portasystemic encephalopathy (Sherlock et al, 1954) and oesophageal variceal haemorrhage (Teres et al, 1976) have been described as the most important complications of portasystemic shunting.

Despite their clinical significance, oesophageal varices constitute only a part of the extrahepatic collateral circulation, and since the method described in this thesis quantitates the total amount of blood bypassing functioning hepatocytes, be it through extrahepatic and intrahepatic shunts, they have little relevance in the context of this thesis. Thus they will be dealt with only briefly. Hepatic encephalopathy however, is thought to be in part the result of the diversion of gut derived compounds and gastrointesinal hormones around functioning hepatocytes. The quantitation of portasystemic shunting is therefore potentially most useful in examining hepatic encephalopathy, in as much as an increase or change in portasystemic shunting may parallel the appearance and progression of hepatic encephalopathy.

(a) Oesophageal varices

One of the major complication of portasystemic shunting is haemorrhage from oesophagogastric varices. Oesophagogastric varices due to cirrhosis of the liver

has been known and documented for many years (Preble, 1900). Portal hypertension induces the development of various types of portasystemic collaterals, although usually a single type of collateral circulation is predominant (Jackson, 1963), thus the diversion of blood into the diaphragmo-oesophageal veins of the caval system leads to varicosities in the submucous layer of the lower end of the oesophagus and upper part of the stomach.

Tamiya and Thal (1960) created oesophageal varices in dogs only when portal or splenic obstruction was associated with an increased flow into the portal system produced by an arteriovenous shunt. Such findings suggest that an increased portal venous flow may be equally as important as the obstructive factor in the pathogenesis of oesophageal varices.

Endoscopy has been predominately used to evaluate the origin of bleeding (Conn and Brodoff, 1964) and numerous techniques allow visualisation of the portal venous system and so the varices (Rousselot and Burchell, 1969).

Contradictory reports however, have been published regarding the relationship between portal hypertension and the severity of the oesophageal varices. Willoughby et al (1964) utilising hepatic vein catherterization found a correlation between the size of oesophageal varices and the severity of portal hypertension. Joly

et al (1971) by transumbilical catheterization of the portal vein, demonstrated as similar relationship. In contrast, Lebrec et al (1980) demonstrated that the presence and size of oesophageal varices were not related to the degree of portal hypertension, an observation further corroberated by splenic pulp manometry (Greene et al, 1965; Palmer, 1953). Although the mechanism of variceal rupture is unclear, it would appear that the height of the portal blood pressure is important (Orloff et al, 1963), and indeed bleeding varices have only been observed if significant portal hypertension exists (Viallet et al, 1975). Further, overall risk of gastrointestinal bleeding was much higher in patients with large sized oesophageal varices than those with no visible or small sized varices (Lebrec et al, 1980), a finding which is in accordance with observations made by Baker et al (1959).

(b) Hepatic encephalopathy

Hepatic encephalopathy and the factors precipitating its development are of particular relevance to this thesis, therefore a comprehesive discussion on hepatic encephalopathy is given.
Portasystemic encephalopathy is to a large extent secondary to the more subtle consequences of the altered circulation which present in the form of

hormonal, nutritional, immunological and pharmacological disorders. The condition is thus thought to be the product of cerebral intoxication secondary to the metabolic changes arising in patients with cirrhosis (Sherlock et al, 1954) or in patients with portacaval anastomosis (McDermott and Adams, 1954), and resulting in behaviour, personality and mood disorders before progressing to coma (Hoyumpa et al, 1979). Immunological and pharamacological disorders do not appear to play a role in the development of hepatic encephalopathy, but they do, however, give rise to humoral and cell mediated immunity abnormalities and enhanced drug bioavailabiltiy.

The combination of both nutritional and hormonal disorders and the onset of portasystemic encephalopathy are extensively interrelated.

(i) Nutritional disorders

Elevated levels of ammonia secondary to an altered nitrogen metabolism have been described in humans with cirrhosis (Bessman et al, 1955), rats with portacaval shunt (Masland, 1964) and cirrhosis (Yamamoto et al, 1987) and patients with portasystemic encephalopathy (Sherlock et al, 1954). Other species have also been observed to develop diffuse neurological abnormalities either spontaneously or when challenged with high protein, orally administered ammonium chloride, or

ammonium-releasing resin (Bollman, 1961).

Pharmacologically active amines have also been implicated in hepatic coma. Raised blood mercaptan levels produced by intestinal methionine metabolism are found in portasystemic encephalopathy, but not in all patients (McLain et al, 1980) and tryptophan is increased in the brain in experimental hepatic coma as well as the cerebrospinal fluid of patients with hepatic coma (Ono et al, 1978). Its metabolism to serotonin has profound effects on the central nervous system. A similar picture to ammonia and amine intoxication can be induced in patients with a high protein diet or when challenged with urea (Phear et al, 1955), or methionine (Phillips et al, 1952; Phear et al, 1956).

Further nutritional aberrations present in the form of elevated tyrosine levels, which are further accentuated by abnormalities in plasma amino acid concentrations arising from hormonal alterations. In 1978 Faraj et al, demonstrated an impaired tyrosine metabolism in dogs with portacaval shunt, elevated tyrosine levels becoming more pronounced as they manifested symptoms of hepatic encephalopathy. Similar results were reported by Ali et al (1980), who attributed decreased tyrosine metabolism to a reduced nutrient hepatic flow, rather than a loss of the liver catabolising enzyme. Such elevated tyrosine levels have been hypothesised to

produce false neurological transmitters, thus interfering with normal synaptic transmission, and so contributing to the symptoms of hepatic encephalopathy (Fischer and Baldessarini, 1971).

Alterations in fatty acid metabolism, shown by elevated concentrations of short and long chain fatty acids have been found in hepatic coma and are believed to act synergistically with ammonia, although a direct association between encephalopathy and fatty acids has not been established (Wilcox et al, 1978).

(ii) Hormonal disorders

The inter-relationship between hormonal alterations and hepatic encephalopathy occurs via abnormalities in plasma amino acid concentrations which have been reported in humans (Fischer et al, 1974 and 1976; Morgan et al, 1978), dogs (Aguirre et al, 1974; Fischer et al, 1975) and rats (Cummings et al, 1976; Rosen et al, 1978) with hepatic cirrhosis or after portacaval shunt.

These abnormalities are characterised by elevated levels of aromatic amino acids, tyrosine, phenylamine and tryptophan as well as methionine and histidine and depressed levels of the branched amino acids, leucine, valine and isoleucine. Such alterations have been attributed to initial hormonal changes, particularly those of the hormone insulin. Hyperinsulinemia together

with glucose intolerance and insulin resistance has been found in patients with cirrhosis (Collins et al, 1969) and in patients after portacaval shunt (Johnson et al, 1977). Hyperinsulinemia is thought to increase catabolism by skeletal muscle and kidney of branched chain amino acids, allowing excess aromatic amino acids to cross the blood brain barrier and so alter neurotransmitter synthesis (Munro et al, 1975), thus precipitating once again the signs and symptoms of hepatic encephalopathy.

Hormonal disturbances such as hyperinsulinemia have been attributed independently to both portasystemic shunting (Johnson et al, 1977) and parenchymal liver damage (Smith-Laing et al, 1979). The contribution of both to the development of hyperinsulinemia has not been assessed. Further, raised levels of glucagon have been evidenced in patients with cirrhosis and portacaval shunt (Alford et al, 1979), and as discussed in a previous section, are thought to be implicated in the increased portal venous inflow. However, the reason underlying the hypersecretion of pancreatic glucagon is as yet unknown.

The following consequences of portasystemic shunting, as already stated do not appear to contribute to the development of hepatic encephalopathy, but they are responsible for the more subtle problems encountered in patients with cirrhosis.

(iii) Immunological disorders

Immunological alterations secondary to portasystemic shunting and cirrhosis present in the form of humoral and cell mediated immunity abnormalities. Evidence of elevated immunoglobulin levels (Prytz et al, 1977) and serum antibodies to gut bacteria (Bjorneboe et al, 1972; Triger et al, 1972) have typified changes in humoral immunity, whereas defects in cell mediated immunity have been shown by diminished delayed hypersensitivity responces (Fox et al, 1969), reduction in peripheral T-cell concentrations (Bernstein et al, 1974) and diminished responses to phytohaemagglutinin (MacSween and Thomas, 1973).

Hypergammaglobulinemia evidenced by electrophoretic studies in cirrhotic livers is the most prevalent raised immunoglobulin (Franklin et al, 1951). Comparable conditions have been created in the rat (Thomas et al, 1976) with the proposition that depressed defects in cell mediated immunity are related in part to the presence of a serum inhibitor (Willems et al, 1969), possibly produced by splenic suppressor cells stimulated by diversion of gut derived antigens through portasystemic shunts (Thomas et al, 1976).

Raised immunoglobulin and antibody concentrations have

been demonstrated both in the presence of patients with portacaval shunt (Galbraith et al, 1976) and rats (Keraan et al, 1974). In contrast, Thomas et al (1976) working on rats and Webb et al (1980) studying patients attributed hepatocellular damage, rather than extrahepatic portasystemic shunting as the influencing factor in the altered humoral immune response. Further, researchers have proposed that the presence of intrahepatic shunts (Prytz et al, 1977) as opposed to extrahepatic shunts are more significant in the causation of the elevated levels of the immunoglobulins and Pomier-Layrargues et al (1980) attributed alterations in kupffer cell uptake secondary to intrahepatic portasystemic shunting.

(iv) Pharmacological disorders

Pharmacological changes surface in patients with liver cirrhosis in the form of enhanced bioavailability of drugs, particularly those drugs which characteristically have a high first pass hepatic clearance (Blaschke and Rubin, 1979). Early on it was accepted that patients with hepatic disease had more central nervous system depression from analgesics and sedative agents than patients with normal liver function (Laidlaw et al, 1961; Schenker et al, 1975) and mounting evidence implied that increased sensitivity in patients with liver disease was due to

altered drug disposition (Wilkinson et al, 1975; Pessayre et al, 1978). Shand and Rangno (1972) were one of the first to observe a marked increase in systemic availability of orally administered propranolol in a patient with a portacaval shunt and further studies by Gugler et al (1975) on dogs with a surgically constructed portacaval shunt resulted in enhanced bioavalability of drugs such as lidocaine and salicylamide. Neal et al (1979) further demonstrated decreased clearance of analgesics, and correlated enhanced bioavailability to drugs with highest hepatic clearances in cirrhotic patients with portasystemic shunting. However, Pessayre et al (1978) and Roberts et al (1978) attributed enhanced drug bioavailavility in cirrhotic patients to an impaired ability of the liver to primarily remove the drug from the blood due to liver damage and secondly to a reduced liver blood flow. It is now accepted that a reduced extraction in cirrhosis will be compounded by the development of mesenteric portacaval anastomoses (Shand, 1979).

MEASUREMENT OF PORTASYSTEMIC SHUNTING

(a) Visualisation of portasystemic shunting

The methods available by which a patient with clinical liver disease could be studied was for many years limited to tests of hepatic function and tissue biopsy. The presence and site of extrahepatic obstruction was also not easily determined by available clinical methods, even at the time of exploratory laparotomy. The development and implementation of visual technology and splenic radiographic injection techniques in the middle of the 1940's (Whipple, 1945; Blakemore and Lord, 1945) allowed more indepth evidence to be gathered on the portal venous system, collateral circulation, and the level of portal venous pressure. During the 1950's and 1960's, portal venography, portal portography, retrograde portography, transcapillary portography and splenic portography were used to radiologically visualise the portal venous system by use of contrast medium injected at various areas depending on the technique being used (Rousselot et al, 1953; Bahnson et al, 1953; Child et al, 1951 and Rousselot and Burchell, 1969). These techniques were used as a means of diagnostically assessing the anatomical location, caliber, patency and haemodynamic derangement of the portal vein and its collateral

circulation, the knowledge of which was used to grade the collateral circulation.

(b) Measurement of portasystemic shunting

The first objective method designed to quantitate the portal venous collateral circulation in cirrhosis was described in 1960 by Iber and co-workers. The method, validated by Reichman et al (1958), was based upon indicator dilution techniques and the use of a radioactive indicator substance, human radioiodinated serum albumin. Collateral flow was estimated from the difference between the measured concentration of iodinated albumin injected into the spleen appearing at the hepatic vein and in the peripheral circulation. Values for portal collateral loss ranged from 0-100% in patients with various forms of liver disease. A similar study carried out two years later by Caesar et al (1962) produced comparable results.

The method used by Iber and colleagues (1960) to assess portasystemic collateral flow was, however, criticised by investigators Nakamura et al (1962). While themselves describing two methods in the measurement of extrahepatic shunted blood flow in liver cirrhosis, Nakamura et al (1962) claimed that calculation of extrahepatic collateral flow would be grossly

overestimated if the described method by Iber et al (1960) was used. The methods used by Nakamura and colleagues (1962) to calculate extrahepatic shunted blood flow produced significantly lower values than those acheived by Iber et al (1960), and these values were claimed by the investigators to be a more realistic indication of shunted blood flow.

The methods used by Nakamura et al (1962) are in themselves, however, subject to critisism. Their first method involved determining continuously the radioactivity of brachial arterial blood after injection of radioactive iodinated serum albumin into the spleen. From the resultant curve of radioactivity the extrahepatic shunted blood flow was calculated as percent extrahepatic shunt. The method however, suffers from an inability to differentiate between curves produced by extrahepatic shunted blood flow and that caused by the blood flow through the liver in the radioactivity curve as well as a failure to record continuous arterial concentrations, thus extrahepatic shunted blood flow could not be quantitatively calculated. In their second method, indocyanine green and radioactive iodinated human serum albumin were injected into the peripheral vein and spleen, and percent extrahepatic shunt was calculated from the dye concentration curves recorded with an ear densitometer and from final concentrations of radioactive iodinated

human serum albumin. The second method appeared to be more sensitive than the first but was still subject to theoretical problems and inaccuracies that beset all applications of indicator dilution techniques utilising splenic injection of a radioactive indicator. Such inaccuracies, as detailed below, have thus limited their application,

(1) The method provides an estimation only of shunting of splenic blood flow, since some or all of the injected label may escape through splenic vein collaterals before mixing with the intestinal flow. Thus independent shunting from the mesentery is disregarded and collateral flow is overestimated.

(2) The indicator injected into the spleen does not all gain quick access to the circulation and delayed absorption or inconstant residual at the injection site introduces a complication in calculating shunt flow (Reichman et al, 1958).

(3) Hepatocellular function must be adequate to allow measurement of hepatic blood flow by an independent clearance method (Reynolds, 1969).

(4) Finally, splenic puncture is contraindicated in the presence of ascites or clotting disorders, both of which are common in patients with liver disease (Rousselot and Burchell, 1969).

It was not until 1972 that a technique which allowed separate evaluation of collateral flow arising from the

splenic and mesenteric portal inflow by means of selective percutaneous cathererization and single injections of I^{131} labelled human serum directly into the respective arteries was described by Groszmann and associates (1972). The technique, previously validated by Cohn et al (1972), allowed the fraction of portal venous inflow shunted through portasystemic collaterals to be computed by comparing the area of an isotope dilution curve recorded from the hepatic vein after injection into the superior mesenteric or splenic artery with that after injection into the hepatic artery. From such studies Groszmann et al (1972) found that up to 62% of mesenteric and 80% of splenic blood flow may undergo extrahepatic shunting in patients with alcholic liver disease. Furthermore, it appeared that shunts developed independently in the mesenteric and splenic bed. However, on interpretating Groszmann et al's, data it must be remembered that shunting has been calculated as a percentage of splenic or mesenteric inflow and that the actual flow in the collateral channels cannot be quantitated because it is dependent on the total splenic and mesenteric flow.

Further studies carried out by Ueda et al (1967) and Okuda and colleagues (1977), attempted to quantitate seperately intra and extrahepatic shunts in one procedure. Previous techniques had allowed only for the seperate evaluation of both types of shunting within

different procedures. Extrahepatic shunts being determined by methods already described and those arising within a diseased liver have been delineated by Shaldon et al, 1961 and Nakamura et al, 1961. However, the method by which an extrahepatic shunt index is calculated by Ueda et al (1967) and Okuda et al (1977) arises from the administration of I^{131}-macroaggregated albumin and 99mTc-macroaggregated albumin respectively, into the splenic vein at the splenic hilum, which in itself is subect to the already mentioned model errors. Further, subsequent errors arise with the inhomogeneity of the macroaggregeted albumin, thus the technique suffers from several possible sources of error.

Despite the development of techniques to measure the proportion of portal blood that bypasses the liver to reach the systemic circulation, no single method has gained practical use due to their complexity, model restrictions and more importantly the fact that all techniques suffer from being highly invasive, (Okuda et al, 1977; Groszmann et al, 1972).

The first hypothetical non-invasive kinetic approach to the estimation of hepatic shunting in chronic liver disease was described in 1979 by McLean and coworkers. The method defines the relationship between the degree of shunting of total liver blood flow around the fraction of functional liver cell mass and develops pharmacokinetic equations to permit the estimation of

both total hepatic blood flow and the extent to which this blood flow is shunted. The method requires determination of the systemic clearance of a high and low hepatic extraction ratio drug in the same patient, indocyanine and antipyrine being used respectively. By application of the developed equations to literature data obtained from patients with moderate or severe chronic liver disease and from patients with a portacaval shunt, McLean et al (1979) were able to demostrate a significant degree of shunting, 27% in patients with moderate chronic liver disease and extensive shunting, 72% in patients with severe chronic liver disease. The basis for the pharmacokinetic method however, requires adequately functioning hepatocytes, thus in a diseased liver deviations from this result, and in a given patient will introduce errors in the estimation of liver blood flow and the degree of shunting. Calculations by McLean et al (1979) have however, been consistent with published data (Bradley et al, 1952; Caesar et al, 1961; Groszmann et al, 1972), thus the pharmacokinetic method appears to be a feasible technique. Its main fault lies in the fact that it is time consuming.

Porchet and Bircher in 1982 outlined the first attempt at a practical non-invasive procedure that approached quantitative assessment of portasystemic shunting. The authors proposed that the systemic availability of oral

glyceryl trinitrate was a measure of the fraction of portal blood bypassing the liver through portasystemic shunts. It was assumed that at low oral doses intestinal absorption of glyceryl trinitrate was complete and hepatic extraction was almost 100 percent, therefore systemic spillover mainly depends on intra or extrahepatic shunting, thus the systemic clearance of glyceryl trinitrate should directly reflect the amount of blood flowing through functioning hepatic parenchyma, and glyceryl trinitrate bioavailability should indicate the fraction of portal blood bypassing it.

It must be stressed that the methods used by Porchet and Bircher (1982) and McLean et al (1979) to quantitate the magnitude of portasytemic shunting do not distinguish between anatomical shunting through large calibre extrahepatic and intrahepatic collaterals and functional shunting as a result of impaired hepatocyte function. The increased bioavailability of gut derived agents thought to be responsible for hepatic encephalopathy arises not only because of the diversion of portal venous blood through major collateral channels, but also because functional damage to the liver reduces its capacity to eliminate these agents on passage through the sinusoids. Any attempt to relate the degree of shunting to the development of encephalopathy must therefore take into

account the latter process i.e. functional shunting, as well as the former. The use of a test substance whose bioavailability reflects both potential types of shunting is therefore the most appropriate choice for this purpose.

In concurrence with the non-invasive principle of the method described by Porchet and Bircher (1982), blood sampling was replaced by digital plethysmography which assessed the pharmacological effects of glyceryl trinitrate, this in turn could be translated into a measure of the systemic availability of the drug. Dose-response curves resulting from graded intravenous infusions of glyceryl trinitrate were used as standard of comparison for the pharmacological response resulting from an oral dose of the drug. Small oral doses were used to achieve a practically complete elimination in normal subjects on first pass and avoid saturation kinetics. Therefore first-order kinetics were assumed. Results demonstrated that normal volunteers had a systemic availability of oral glyceryl trinitrate of 2-4 percent, while patients with cirrhosis of the liver showed variation between 15 percent and 85 percent.

Problems within the method however arose relating to digital plethysmography which proved to be subject to large measurement errors. Also drug absorption through oral or oesophageal mucosa may also bypass the liver

and simulate portasystemic shunting. Further, glyceryl trinitrate maybe subject to first-pass elimination in the intestine and retention within the tubing during the intravenous infusion, thereby installing errors into the calculation of infusion rates and systemic availabilities. More importantly glyceryl trinitrate may also modify the splanchnic circulation and thereby potentially change the degree of shunting.

More recently Cavanna et al (1987) used the compound sorbitol, already proven to have a high hepatic elimination (Molino et al, 1987) for non-invasive assessment of the magnitude of portasystmic shunting. First-order kinetics were again assumed and by arterial sampling and enzmymatic spectrophotometric analysis sorbitol concentration in plasma were determined. The ratio between the area under the curve from an intravenous infusion of sorbitol and that from orally administered sorbitol were used in the calculation of sorbitol bioavailability.

The bioavailability of sorbitol, as with glyceryl trinitrate, reflects both anatomical and functional shunting. Normal subjects exhibited zero sorbitol bioavailability whereas patients with cirrhosis and portacaval shunts demonstrated varying bioavailabilities. Despite patients with portacaval shunt presumably having 100 percent shunting, sorbitol bioavailability was only able to demonstrate 38 percent

shunting in such patients. The failure of sorbitol to measure accurately shunting may be due to its elimination across the intestinal wall during absorption.

The methods described by Porchet and Bircher (982) and Cavanna et al (1987), however were well accepted by patients with the minimum of inconvenience and demonstrated a realistic means of determining portasystemic shunting.

Indeed it is the theory behind these two techniques that underlies the method described in this thesis. The choice of galactose as a test substance, as discussed in the following section, lies in the fact that galactose does not undergo intestinal elimination and has a high hepatic elimination.

AIMS OF THESIS

1. To develop a theoretical model to describe the behaviour of an oral test substance that could be used to quantify the magnitude of portasystemic shunting, which in this study represents anatomical and functional shunting combined.

2. To test the validity of the model using galactose as a test substance in humans and in a rat model of prehepatic portal hypertension. Independent quantitation of the magnitude of portasystemic shunting by radioactive microspheres will also be carried out in the rat model.

3. To measure the systemic availability of oral galactose in control patients and patients with documented portal hypertension and portasystemic shunting.

CHAPTER TWO GALACTOSE ELIMINATION KINETICS AND THE MEASUREMENT OF LIVER FUNCTION, LIVER BLOOD FLOW, AND PORTASYSTEMIC SHUNTING

INTRODUCTION

SECTION A. GALACTOSE CLEARANCE AS A TEST OF LIVER FUNCTION

1. Theory
2. Discussion

SECTION B. GALACTOSE CLEARANCE IN THE MEASUREMENT OF LIVER BLOOD FLOW

1. Theory
2. Discussion

SECTION C. ORAL GALACTOSE AND THE ASSESSMENT OF PORTASYSTEMIC SHUNTING

1. Theory
2. Discussion

INTRODUCTION

Hepatic elimination of galactose from the body has been proposed as the basis of a test of liver function (Colcher et al, 1946; Tygstrup, 1964 and 1966), of a method of measuring liver blood flow (Henderson et al, 1982), and in this thesis of a means of assessing the magnitude of portasystemic shunting.

Galactose elimination from the circulation by the liver is an active metabolic process with a maximum limiting rate (Vmax) dependent upon the phosphorylation of galactose to galactose-1-phosphate by the enzyme galactokinase, one of several enzymes in the Leloir metabolic pathway responsible for the interconversion of galactose to glucose (Cohn and Segal, 1973: Goresky et al, 1973). It has been shown that galactose elimination may be described by a mathematical kinetic model based on the assumption that hepatocytes eliminate the substrate from the body by an irreversible process described by Michaelis-Menten kinetics (Keiding et al, 1976). The Michaelis-Menten equation $V = VmaxCa/(Km+Ca)$ describes the elimination rate, V, of galactose at a given concentration Ca. The kinetic parameters, Vmax and Km can be estimated from the mathematical model providing a direct value of the maximum galactose elimination rate, and galactose concentrations which give half the maximum elimination

rate respectively.

The knowledge of Vmax and Km can be applied in the design of methods of measuring liver function, liver blood flow and portasystemic shunting. A test of liver function can be made when the concentration of galactose, Ca, is greater than Km and the elimination rate of galactose is zero-order and approximates Vmax. Conversely, when the concentration of galactose is less than Km the elimination rate of galactose is first-order and equals VmaxCa/Km and conditions are suitable for measuring blood flow and portasystemic shunting.

Michaelis-Menten kinetics are applicable not only at the cellular (hepatocyte) level but also to the liver as a whole (Keiding, 1973). In this chapter whole organ kinetics are considered in the hepatic elimination of galactose. A more detailed discussion of Michaelis-Menten kinetics at the cellular level will be given in a subsequent chapter.

This Chapter, which is divided into three sections, examines in detail the theory and assumptions underlying the techniques of applying galactose as a test of liver function, and a measure of hepatic blood flow. This theory is then extended to develop a theoretical model for a method of assessing portasystemic shunting non-invasively in man. This model in turn has been applied in man, the results of which are discussed in Chapter 4.

SECTION A. GALACTOSE CLEARANCE AS A TEST OF LIVER FUNCTION

THEORY

Before devising formulae to fit the theoretical model for clearance of blood galactose as a test of liver function, a number of assumptions must be made, these being;

1). Galactose is eliminated from blood by the liver via an active metabolic process which has a maximum limiting rate, Vmax, dependent on liver function. Saturation of this metabolic pathway occurs if blood galactose concentration (Ca) exceeds a certain threshold level, when the rate of hepatic elimination is independent of galactose concentration.

2). Exchange of galactose between blood and other body compartments is sufficiently rapid that blood galactose can be considered to be in continuous equilibrium with total body galactose, i.e. the rate of disappearance of galactose from blood accurately reflects the rate of elimination from the body as a whole.

3). Elimination of galactose from the body by other routes either is negligible or can be quantified.

From the above assumptions the following formulae can be derived, the application of which allow, Vmax, the maximum galactose elimination rate, to be determined experimentally.

If extrahepatic elimination is for the moment neglected (assumption 3), then, following the intravenous administration of a large quantity of galactose (Qo), typically 500 mg/kg body weight (Tygstrup, 1964), the amount remaining in the body after time t is given by,

$$Q(t) = Qo - Vmax.t \quad \text{.....2.1}$$

From the second assumption above it is implied that blood galactose concentration (Ca) is proportional to Q(t), and can be written,

$$Ca(t) = Q(t)/Vd = Cao - (Vmax/Vd).t \quad \text{.....2.2}$$

where Vd is the volume of distribution of galactose, and Cao, the hypothetical concentration in blood immediately after injection, is given by,

$$Cao = Qo/Vd \quad \text{.....2.3}$$

Schematically illustrated in Figure 1 is the typical form of the systemic blood galactose concentration time curve observed experimentally after a single, rapid injection of galactose (Tygstrup and Winkler, 1954). The rapid fall in concentration immediately after injection in part reflects diffusion of galactose out of the vascular bed. The gradual decline of the curve i.e. the linear clearance of galactose thereafter reflects the rate of hepatic elimination. As equilibration of galactose between blood and tissue is

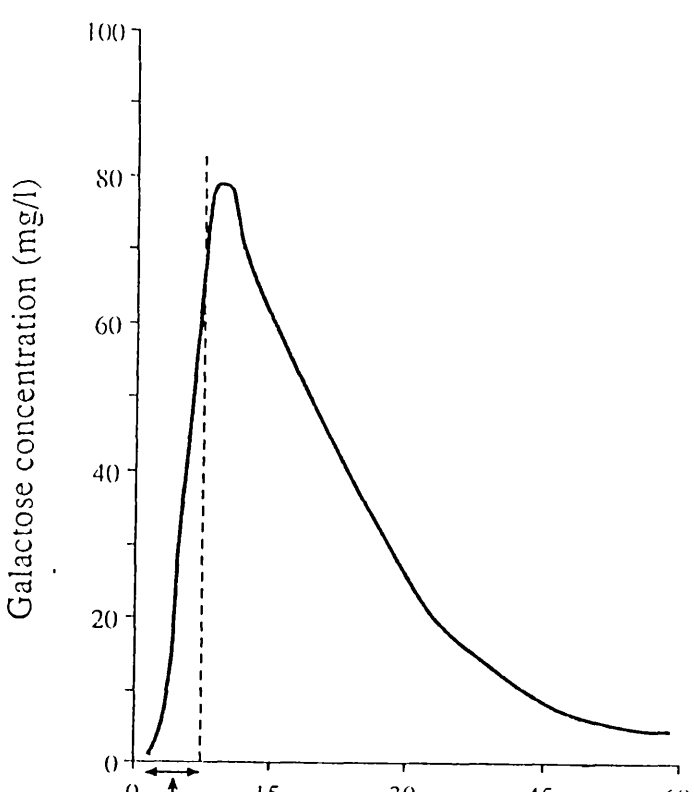


Figure 1

This shows the typical form of a galactose concentration time curve after a single, rapid intravenous injection of galactose. The rapid fall in concentration immediately after injection in part reflects diffusion of galactose out of the vascular bed. The gradual linear decline of the curve thereafter reflects the rate of hepatic elimination, and the non-linear tail of the curve reflects the fall in concentration below the level at which hepatic extraction is saturated.

not in practise instantaneous, then Cao must be determined by extrapolation of the linear part of the curve that follows. Tygstrup (1963) suggested a time shift of the elimination curve of seven minutes as an empirical correction for non-uniform distribution of galactose in this early phase. The tail of the curve is non-linear, reflecting the fall in galactose concentration below the level at which hepatic extraction is saturated.

By measurement of the gradient of the linear phase of the clearance curve (-Vmax/Vd by equation 2.2), estimation of Cao, and knowledge of Qo, Vd, the volume of distribution, and Vmax, the galactose elimination capacity, can be calculated.

This treatment, as already stated, ignores extrahepatic removal of galactose, which is principally by renal excretion (Tygstrup, 1961: McNamara and Segal, 1972). Typically, 10% of the injected dose is recovered in urine, most of it is excreted during the early, non-linear phase of clearance (Tygstrup, 1964). Simple subtraction of the excreted galactose from the quantity injected is therefore an acceptable correction for extrahepatic clearance.

DISCUSSION

Experiments on intravenous administration of galactose as a test of liver function have demonstrated that the amount of galactose eliminated per unit time is constant, independent of concentration within a certain range (Tygstrup and Winkler, 1954). The linear portion of the slope of the blood galactose clearance curve after a single, rapid intravenous injection has been taken as a measure of the maximum galactose elimination rate and measured values are typically in the range 300-600 mg galactose/min in control subjects, and 200-400 mg galactose/min in cirrhotic patients (Tygstrup, 1964; Lindskov et al, 1983). With respect to this study, these values are chiefly of interest for comparison with the galactose infusion rates employed in the techniques to be discussed below (up to 100 mg galactose/min) which are required to be substantially below the maximum elimination rate (Henderson et al, 1982). Indeed, the model for galactose clearance in the measurement of hepatic blood flow is based upon infused galactose concentrations well below the maximum galactose elimination rate (Vmax).

Estimation of the maximum galactose elimination rate from the single injection curve rests on some assumptions which are idealizations of the actual

conditions: the elimination curve in the selected interval is a straight line, urinary loss may be corrected for by the average excretion rate, and uneven distribution of galactose is compensated for by a time shift of the elimination curve of seven minutes (Tygstrup, 1963). Deviations from these assumptions may affect the calculated maximum galactose elimination rate in a direction, and to an extent, which depends on several factors.

It has been suggested, that the maximum galactose elimination rate as estimated from the peripheral curve (Tygstrup, 1964) overestimates liver function as determined from splanchnic galactose uptake, estimated from arterio-hepatic venous difference and splanchnic blood flow following a single intravenous injection (Ranek et al, 1983). A difference of 40% in galactose elimination rate was found between the two methods in patients with cirrhosis (Ranek et al, 1983), and attributed to the redistribution of galactose in the body and elimination outside the splanchnic organs (and excretion by the kidneys, for which the standard equation has a correction).

The existance of an unidentified extrahepatic, extrarenal glactose elimination pathway was challenged by Lindskov et al (1983), who employed a steady-state infusion technique to estimate splanchnic galactose uptake. The technique reduced the influence of

distribution problems, but had its own limitations, for example, errors in methods for determining regional blood flow and the representativity of catheter sampling in the hepatic and renal veins. Lindskov et al (1983) found an almost identical galactose elimination rate to that of Ranek et al (1983), 319 and 313 mg galactose/min respectively in patients with liver disease, but also found that the galactose elimination rate overestimated the splanchnic galactose elmination rate by only 25 percent in patients with cirrhosis. Lindsov et al (1983) concluded that this difference could be accounted for in equal parts to uneven distribution and by elimination in the kidneys, which had previously been underestimated by the conventional correction for urinary loss. This elimination may be partly excretion in the urine, partly intrarenal conversion.

The large extrahepatic-extrarenal galactose elimination pathway, as estimated after single injection in the study by Ranek et al (1983) can be thus attributed to over estimation of the total body galactose elimination rate and underestimation of splanchnic galactose uptake due to distribution phenomena. However, extrahepatic-extrarenal conversion of galactose cannot be excluded, but is estimated by Lindskov et al (1983) not to exceed 36 mg galactose/min.

It must be pointed out, that galactose elimination outside the splanchnic organs does not invalidate the galactose elimination rate as a quantitative liver function test, especially when used repeatedly in the same patient because the extrahepatic, extrarenal elimination tends to be constant.

SECTION B. GALACTOSE CLEARANCE IN THE MEASUREMENT OF LIVER BLOOD FLOW

THEORY

Experimental measurements of hepatic blood flow are based upon application of the Fick principle (Bradley et al, 1945; Waldstein and Arcilla, 1958). According to the Fick principle, the rate of extraction of any substance from blood by an organ is given by,

$$V = F.(Ca-Cv) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2.4$$

where V is the rate of extraction, F is organ blood flow, Ca is the concentration of the substance in arterial blood, and Cv the concentration in venous blood draining from the organ. The liver is a special case because of its dual blood supply, but, if extraction of the substance by organs draining into the portal vein is negligible, portal venous concentration is equivalent to arterial or peripheral venous concentration, and the Fick principle may be applied to the liver by equating F with total liver blood flow and Cv with hepatic venous concentration. Equation 2.4 has been widely used to measure hepatic blood flow via the clearance of circulating dyes such as indocyanine green, with sampling from peripheral blood and from the hepatic veins (Caesar et al, 1961; Leevy et al, 1962). In experiments with both peripheral and hepatic venous

sampling of blood galactose, Ranek et al (1983) and Lindskov et al (1983) observed that, during the linear clearance phase described in the preceding section, a constant difference was maintained between Ca and Cv, in keeping with the assumption of constant (saturated) clearance. However, the linear phase ended with the falling of the hepatic venous concentration effectively to zero, after which the peripheral concentration declined exponentially (Figure 2). Clearance of galactose in this phase is described as a first-order process, the metabolic removal pathway for galactose in the liver is nolonger saturated and the exponential decline in blood concentration with time depends on liver blood flow.

The observation that, for sufficiently low values of galactose concentration (Ca), hepatic extraction of galactose was essentially complete was a crucial one, as it implied that galactose could be used for the measurement of liver blood flow without hepatic venous sampling (Tygstrup and Winkler, 1958). Later work, to be discussed below, has defined more clearly the limitations of the assumption of complete extraction - it is manifestly invalid in the presence of liver disease, for example, as a result of an alteration in perfusion together with a reduced hepatocellular function - but we first continue on the basis that it is a good approximation.

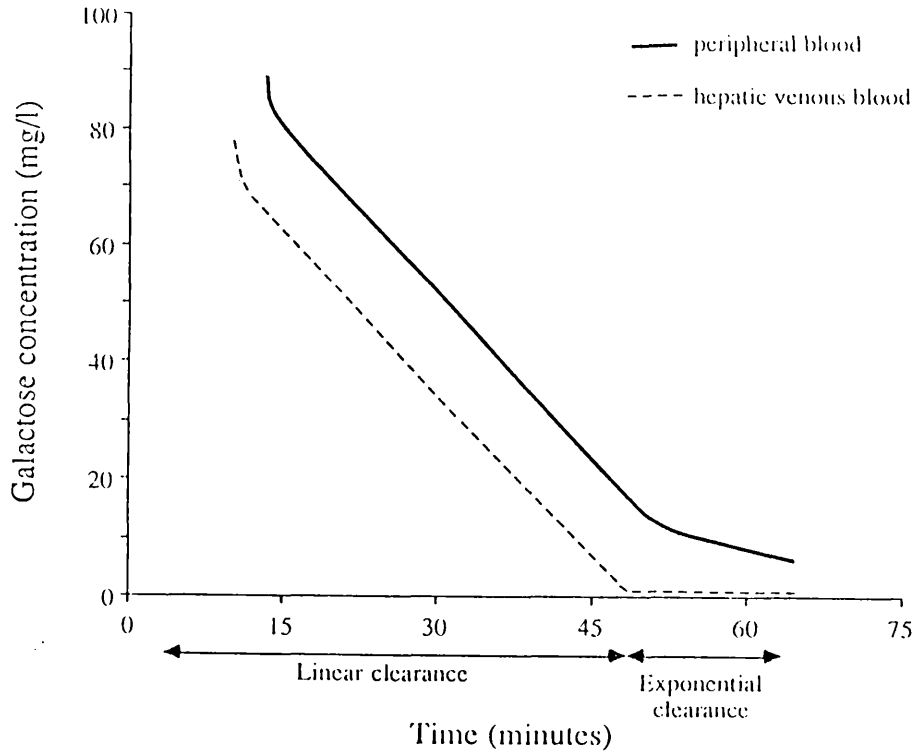


Figure 2

This shows both linear and exponential clearance phases of galactose following a single rapid intravenous injection of galactose. Linear clearance of galactose ends as hepatic venous concentration falls to zero, systemic galactose concentration thereafter declines exponentially. The observation that, for sufficiently low values of galactose concentration, hepatic extraction of galactose was essentially complete, implies that galactose could be used for the measurement of liver blood flow without hepatic venous sampling.

One possible approach to the measurement of liver blood flow is through the analysis of the exponentially decaying peripheral galactose concentration curve. Adopting the notation of the previous section, together with the assumptions of "instantaneous" equilibration and negligible extrahepatic extraction, the rate of extraction (V) can be expressed as,

$$V = -dQ(t)/dt = -Vd.dCa(t)/dt \quad \ldots\ldots\ldots\ldots\ldots\ldots 2.5$$

where, Vd = volume of distribution
Ca(t) = peripheral galactose concentration at time t

Combining equations 2.4 and 2.5, and setting Cv to zero, gives,

$$dCa(t)/dt = -(F/Vd).Ca(t) \quad \ldots\ldots\ldots\ldots\ldots\ldots 2.6$$

Equation 2.6 has the solution,

$$Ca(t) = Cao.exp(-F.t/Vd) \quad \ldots\ldots\ldots\ldots\ldots\ldots 2.7$$

Where, Cao = peripheral galactose concentration immediately after intravenous injection

Hepatic blood flow (F) can be determined by measurement of the exponential decay constant and estimation of Vd as described above. However, this approach has the disadvantages that it depends on (a) accurate measurement of a rapidly decaying concentration curve, (b) the assumption of tissue/blood equilibration on

a short time scale, and (c) estimation of Vd.

The alternative approach, circumventing these problems, is to infuse galactose peripherally at a known, constant rate (I). Provided that I < Vmax (i.e. galactose elimination is first-order), blood galactose concentration will eventually reach an equilibrium level (Cae), at which the rate of elimination (V) from the body equals the rate of infusion (I). If extrahepatic elimination is negligible, and hepatic venous concentration is truly zero, equation 2.4 becomes,

$$I = F.Cae \quad \text{.....2.8}$$

yielding hepatic blood flow (F) directly from known or easily-measured variables.

To take formal account of incomplete hepatic extraction, the extraction efficiency (E) is defined as,

$$E = (Ca - Cv)/Ca \quad \text{.....2.9}$$

and equation 2.4 becomes,

$$V = E.F.Ca \quad \text{.....2.10}$$

The ratio V/Ca = E.F is termed hepatic clearance, and equals the volume of blood totally cleared of galactose by the liver per unit of time. Unless hepatic venous

blood is sampled, or hepatic extraction efficiency (E) otherwise determined, only clearance can be measured, as an approximation to flow. If there is significant extrahepatic elimination of galactose, the rate of infusion at equilibrium equals the sum of the hepatic and extrahepatic elimination rates, and clearance can likewise be separated into hepatic and extrahapatic components.

DISCUSSION

The most comprehensive study of galactose clearance in patients at a low concentration range and at a constant intravenous infusion rate has been performed by Henderson et al (1982). In their study galactose was infused at a rate of 50 mg galactose/min and 40 mg galactose/min in normal and cirrhotic patients respectively. This rate of infusion was well below the maximum hepatic elimination rate (Vmax) for galactose of 300-600 mg galactose/min and 200-400 mg galactose/min, found in normal and cirrhotic patients as previously discussed in section A. A similar study was performed by Tygstrup and Winkler in 1958 in which galactose was infused in control subjects and patients with liver disease at a rate no greater than 300 mg galactose/min. Galactose clearance was calculated by both investigators as the infusion rate divided by the mean galactose concentration in the peripheral venous blood during the measurement period. The mean galactose clearance values found by Tygstrup and Winkler (1958) in patients with and without liver disease was 1046 ml/min and 1420 ml/min respectively. Henderson et al (1982) found galactose clearance values in the range 1200-1600 ml/min in control subjects, and 700-950 ml/min in cirrhotic patients. Tygstrup and Winker (1958) and Henderson and colleagues (1982) also

measured hepatic extraction efficiency by hepatic venous sampling. Henderson et al (1982) found mean hepatic extraction efficiency values of 94% for normal subjects and 79% for cirrhotic patients, at infusion rates of 50 mg galactose/min and 40 mg galactose/min respectively. Correspondingly Tygstrup and Winkler (1958) found hepatic extraction efficiency values of 88% and 69% in normal subjects and patients with liver disease respectively. Tygstrup and Winkler (1958) also found good correlation between galactose clearance and hepatic blood flow as measured by the bromsulfalein method in normal patients. From the study by Tygstrup and Winkler (1958) and Henderson et al (1982) it was not possible, however, to determine whether the lower extraction efficiency in cirrhotic patients was due to intrahepatic portasystemic shunts or to impaired hepatocyte function.

Further, Henderson et al (1982) measured urinary excretion and erythrocyte metabolism, and estimated each to contribute only 2% to total clearance. A similar study to that by Henderson et al (1982), carried out the following year (Henderson and Hanna, 1983) produced comparable results, and led both groups of investigators to conclude that galactose clearance at low concentrations was a useful estimate of functional liver blood flow. It must be borne in mind however, that hepatic galactose clearance is a measure

of hepatic blood flow (F) only if hepatic extraction efficiency (E) is known i.e. Clearance = E.F. Unless hepatic venous blood is sampled, only clearance can be determined as an approximation to flow. A reduction in hepatic extraction efficiency will occur in liver disease but to what extent galactose clearance accurately reflects hepatic blood flow in the absence of a hepatic extraction efficiency measurement is debatable (Groszmann, 1983).

Galactose clearance as a measure of hepatic blood flow has been extended to dogs (Hanna, 1984) and to a procedure using a single injection technique and higher blood galactose concentrations in humans (Zaramella et al, 1985). Results by Zaramella et al (1985) were superimposible to values obtained for hepatic galactose clearance by Tygstrup and Winkler (1958), and a close correlation between galactose clearance and hepatic blood flow as determined by bromosulfalein infusion was found in patients with various degrees of hepatic function.

It has been argued however, by Keiding and Bass (1983) that the hepatic elimination capacity of galactose is not high enough to ensure satisfactory approximation of clearance to hepatic blood flow in subjects with liver impairment (Ranek et al, 1976), although in subjects with no liver disease hepatic clearance approximates liver blood flow. The view that galactose clearance is

a useful measure of functional liver blood flow has also been challenged by Keiding (1988), who performed a comparative study of galactose and indocyanine green (ICG), which were infused simultaneously in normal subjects. Systemic clearance of galactose was found to overestimate by 33% the value of liver blood flow as measured with ICG by the Fick principle (equation 2.4), regarded by the author as the gold standard by which hepatic blood flow should be determined.

Hepatic extraction efficiency was 91%, in reasonable agreement with Henderson's value of 94%, and too high to account for the discrepancy between clearance and blood flow. Urinary excretion was found to be negligible, and Keiding (1988) concluded that there must be a further, unidentified extrahepatic clearance mechanism, possibly renal metabolism. However, Dahn et al (1988), who performed a similar study in septic patients and normal volunteers, found good agreement between galactose clearance and ICG measurements of liver blood flow in the latter group, and a discrepancy in sepsis that could be fully accounted for by reduced hepatic extraction efficiency for galactose in this group. The existence of a significant extrahepatic metabolic pathway for galactose, and the accuracy of galactose clearance as a measure of liver blood flow, therefore remain controversial questions at present.

SECTION C. ORAL GALACTOSE AND THE ASSESSMENT OF PORTASYSTEMIC SHUNTING

THEORY

The tests described so far are based on the introduction of galactose as a bolus injection or continuous infusion into the systemic circulation. It has been found by hepatic venous sampling that circulating galactose, below a certain concentration threshold, is almost completely extracted from blood during a single passage through the normal liver (Henderson et al, 1982). This implies that if galactose were introduced directly into the portal vein, at a concentration which did not exceed the maximum elimination rate (Vmax, from the Michaelis-Menten equation) for galactose by the liver i.e the liver threshold concentration, little would escape metabolism on its first passage through the liver to enter the systemic circulation. In a patient with portasystemic shunting, however, some portal venous galactose would bypass functioning liver tissue through collateral channels, and the remainder might be extracted by the hepatocytes with reduced efficiency because of liver disease. The aim of the proposed technique is to quantify the fraction of galactose that enters the systemic circulation after it is introduced indirectly

into the portal vein by oral administration and absorption from the gastrointestinal tract. No distinction is made between "anatomical shunting" of galactose through large-calibre collateral vessels, and "functional shunting" through poorly functioning liver hepatocytes, as galactose is intended to model the behaviour of gut-derived agents whose bioavailability is increased in similar fashion by both mechanisms.

Two key assumptions are made in the model;

a). The oral dose of galactose is absorbed completely into portal venous blood over the measurement period, without retention or metabolism by the gut.

b). The rate of elimination of recirculating galactose at any instant is proportional to peripheral blood galactose concentration. Recirculating galactose is here distinguished from the galactose that is extracted on first pass through the liver, and its rate of elimination refers to all routes, whether hepatic or extrahepatic.

A schematic diagram of the typical form of a blood galactose concentration time curve recorded after administration of an oral dose of galactose to a patient with portasystemic shunting is shown in Figure 3. The return of the curve to baseline at time T reflects the completion of absorption and elimination, and the curve is assumed to be determined experimentally up to this point. Adopting the

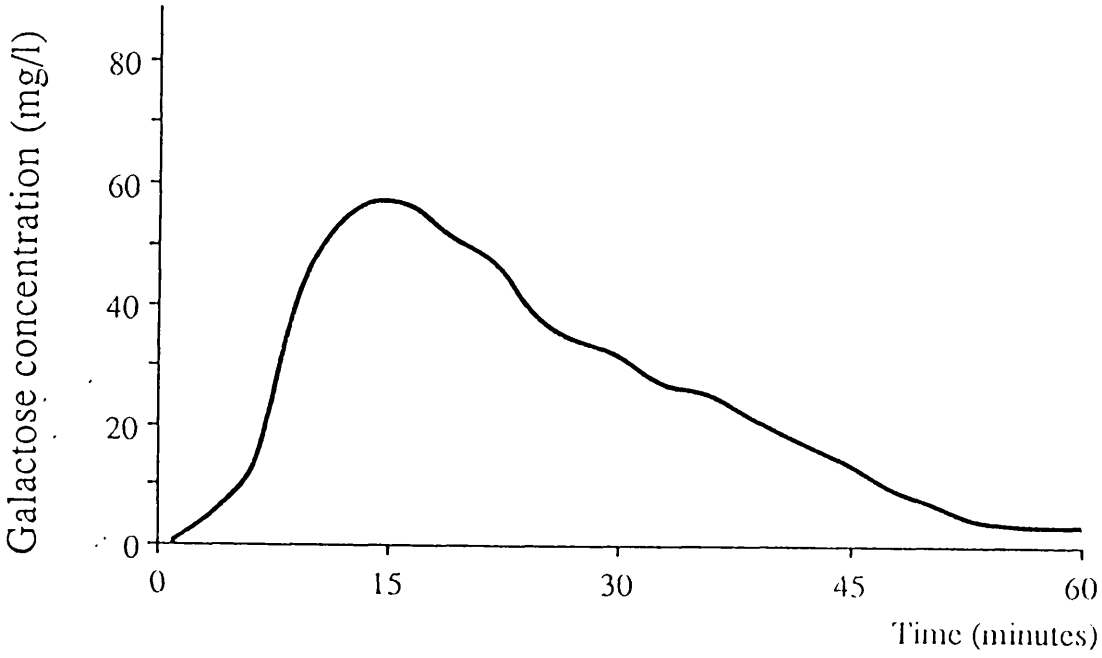


Figure 3

This shows the typical form of a galactose concentration time curve recorded after administration of an oral dose of galactose to a patient with portasystemic shunting. The return of the curve towards the baseline reflects the completion of absorption and elimination of galactose.

notations of the previous section, and by the second assumption above, the rate of elimination of recirculating galactose (V) at any instant is given by,

$$V = K.Ca(t) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2.11$$

where K is a constant equal to total systemic galactose clearance, and Ca(t) represents peripheral galactose concentration at time t. The total amount of recirculating galactose eliminated over time T must equal the quantity that enters the systemic circulation (Qs), and is given by the time integral of V;

$$Qs = \int V.dt = K \int Ca(t).dt = K.AUC \quad \ldots\ldots\ldots\ldots 2.12$$

where, $AUC = \int Ca(t).dt$ represents the area under the curve in Figure 3.

If the oral dose (Q) is completely absorbed, the fraction of portal venous galactose that enters the systemic circulation (SA = systemic availability of galactose) is thus,

$$SA = Qs/Q = K \int Ca(t).dt/Q \quad \ldots\ldots\ldots\ldots\ldots\ldots 2.13$$

K is determined by infusing galactose peripherally at a known rate (I) and measuring the equilibrium value of Ca (Cae), which satisfies

$$I = K.Cae \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2.14$$

Hence, from equations 2.13 and 2.14,

$$SA = Qs/Q = I \int Ca(t).dt \; / \; Q.Cae \quad \ldots\ldots\ldots\ldots\ldots 2.15$$

The systemic availability of galactose in this study is attributable to both anatomical and functional shunting, i.e.

$$SA = S + (1-S)(1-E) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2.16$$

Where S represents anatomical shunting via large calibre collateral vessels, (1-S) represents that fraction of portal blood that enters the liver and (1-E) represents that proportion of galactose which on entering the liver is not extracted by hepatocytes i.e (1-S)(1-E) represents functional shunting. In this method no distinction can be made between the extent to which anatomical and functional shunting each contribute to the systemic availability of galactose. The systemic availability of galactose can thus be readily determined from easily measurable parameters in equation 2.15.

DISCUSSION

In the above analysis, it should be noted that, no assumptions have been about equilibration between blood and tissue of the oral dose of galactose. We have assumed that its rate of elimination by organs of extraction (chiefly the liver and kidneys) is instantaneously proportional to its concentration in blood, but not that it is in equilibrium between blood and its extravascular distribution space. The alternative conceptual approach, that of relating peripheral blood concentration to an "equivalent infusion rate", would yield the same results, but would mask this independence of tissue/blood equilibration. It is necessary to attain equilibrium during the constant infusion of galactose, however, so that the infusion rate can be equated with the elimination rate. It is of no importance what fraction of recirculating galactose is cleared by extrahepatic routes, and therefore unnecessary to measure urinary excretion. However, there must be proportionality between the rate of clearance by all routes and the concentration in blood. Renal excretion of galactose is known to be proportional to concentration (Tygstrup, 1961), and metabolism by red blood cells, although possibly not proportional, is quantitatively negligible (Henderson et al, 1982). The kinetics of the unidentified

extrahepatic route proposed by Keiding (1988) are unknown, but, as discussed above, there is conflicting evidence on the quantitative significance of this route.

If hepatic extraction is complete, proportionality is assured for this route of elimination, but otherwise it follows from equation 2.10 that hepatic extraction efficiency must be independent of blood galactose concentration. Hepatic extraction is a saturable process which has been shown experimentally to obey Michaelis-Menten kinetics (Keiding et al, 1976; Keiding and Chiarantini, 1978), and the extraction efficiency will in fact decrease with increasing concentration. However, there is good evidence that this decrease is small for concentrations within the range actually encountered. Henderson et al, (1982) investigated the effect of doubling the rate of infusion of galactose on measurements of galactose clearance performed as described in the preceding section. They found that, in both normal subjects and patients with cirrhosis of the liver, clearance was only reduced by 8% on average at the higher rate of infusion (50-100 mg galactose/min), implying that hepatic extraction efficiency varied relatively little over concentrations up to 100 mg/l, and that galactose clearance was first-order process.

The incomplete hepatic extraction observed in normal subjects implies that some oral galactose will enter

the systemic circulation in the absence of liver disease and/or portal hypertension, and it is an acceptable limitation of the technique that the measured parameter cannot be equated with portasystemic shunting as generally understood. It is an index which is related to shunting, and which may predict the bioavailability of gut-derived agents which are partly or completely removed on first pass by the liver in the absence of shunting (Bjorneboe et al, 1972). In a later chapter we consider explicitly the Michaelis-Menten equation, and the variability of hepatic extraction efficiency, in the context of experimental studies on galactose elimination kinetics in portal hypertension. The development of the present theoretical framework has been guided by the objective of obtaining useful information from a practicable clinical test, in which simplicity of performance and analysis are at a premium, and which existing evidence suggests will yield acceptably accurate results.

CHAPTER THREE MEASUREMENT OF SYSTEMIC GALACTOSE BY ENZYME ASSAY AND FLUORESCENCE SPECTROPHOTOMETRY

1. Introduction
2. Materials and methods
3. Analytical accuracy and precision
4. Results
5. Discussion

INTRODUCTION

Until recently, work in numerous areas of biological investigation has been restricted by the lack of a convenient and reliable technique for determining the galactose content of biological solutions. Such techniques which have been available have been seriously limited in usefulness and applicability by complexity, lack of specificity, or by a range of sensitivity inadequate to detect the small concentrations frequently encountered (Tygstrup et al, 1954; Date, 1958; Atkinson et al, 1961). This chapter describes one of two currently available assays which allow adequate detection of galactose in the concentration range required in this study. Whole blood galactose and urine galactose concentration levels were determined in all experiments by application of the galactose dehydrogenase enzyme assay (Wallenfels et al, 1962; Rommel et al, 1968) utilising fluorescence spectrophotometry.

The analytical accuracy and precision of the method has been assessed from the standard deviation between duplicate and/or triplicate precipitations of blood from fasted rats after the addition of known quantities of galactose, together with the measured recovery of galactose from these samples.

The recovery of galactose added to blood from fasted

human volunteers and the standard deviation between triplicate precipitations of this blood was similarly assessed.

MATERIALS AND METHODS

Sampling of Blood

A). In all patient studies, as described in Chapter 4, blood was collected in a 1 ml syringe from an indwelling venous catheter in the right arm.
All blood samples were deproteinised immediately after collection to minimise erythrocyte metabolism. 0.1ml of the venous sample was dispensed into a microcentrifuge tube (Sarstedt, West Germany) containing 0.5ml, 0.33mol/L perchloric acid. This was inverted several times to ensure complete mixing.
The deproteinized blood sample was then microcentrifuged (2500rpm, Model 320, Quickfit Instrumentation, England) for three minutes and the supernatant pipetted into a fresh microcentifuge tube and either stored at +4 degrees C for up to three days or frozen at -20 $\pm$ 5 degrees C until analysis.

B). In all animal experiments, male Sprague-Dawley rats weighing 250-390 grams were used. 0.2ml blood samples were collected at various time intervals, as described in Chapter 6, into a 1ml syringe from the right cannulated femoral artery after induction of anaesthesia with 4% halothane in a 2:1 nitrous oxide/oxygen mixture and after a tracheostomy was

performed. Ventilation of the animal was with a Harvard Rat Ventilator (Model 680) as described in chapter 5. 0.1 ml of the arterial blood was added to 0.5 ml perchloric acid, microcentrifuged and stored as described above for patient blood prior to analysis. Normal saline was flushed into the cannula after each blood sample to maintain animal blood volume.

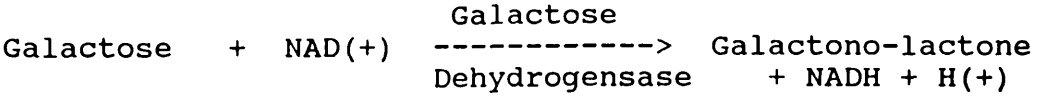
Enzyme Assay and Fluorescence Spectrophotometry

Blood concentrations of galactose were determined by reaction with the specific oxidative enzyme galactose dehydrogenase (EC 1.1.1.48) coupled to an ultraviolet spectrofluorometric measuring technique. The test principle (Figure 4) is based upon the interaction of the enzyme with D-galactose and the co-enzyme nicotinamide-adenine dinucleotide (NAD). The resultant enzyme-substrate interaction converts galactose into galactono-lactone with formation of the fluorescing co-enzyme NADH. The resultant fluorescence is proportional to the amount of galactose present in the original blood or urine sample and was measured by a fluorescence spectrophotometer (Perkin-Elmer MPF-3L), using the following settings;

Excitation wavelength	----------	365nm
Emmission wavelength	----------	460nm
Emmission slit width	----------	3nm
Excitation slit width	----------	20nm
Reference sensitivity	----------	Direct
Response	----------	Fast

Figure 4

Galactose Test Principle



Galactose Calibration Graph

Before blood samples were measured for galactose concentration a standard straight-lined calibration graph of relative fluorescence (millivolts) versus galactose concentration (mg/l) was constructed.

In rat experiments, two calibration concentration ranges of galactose were chosen to allow accurate measurement of galactose at low and high concentrations in blood. The low concentration range consisted of six galactose standards each containing 0, 2, 4, 6, 8, 10 mg/l of galactose respectively. These were prepared from a standard water stock solution of 10 mg/l of galactose with the appropriate dilutions being made in distilled water. The high concentration range of galactose consisted of six galactose standards ranging in concentration from 0, 8, 16, 24, 32, 40 mg/l. These were prepared from a standard water stock solution of 40 mg/l and again the appropriate dilutions were made in distilled water.

For the enzyme assay, 0.2ml of each galactose standard was added to 3.0ml 0.2mol/l, pH7.5 phosphate buffer and 0.10ml 13mmol/l NAD. Galactose dehydrogenase, phosphate buffer and NAD were commercial preparations from Boehringer, Mannheim (F.R.G). This solution was gently mixed, transferred into a 3.5ml cuvette and the fluorescence A1 measured by the fluorescence

spectrophotometer. 0.02ml (>20U/ml) of the enzyme galactose dehydrogenase was then added to each sample, which was rotamixed and left until completion of the reaction, approximately 30 to 40 minutes, when fluorescence A2 was measured. Pilot studies showed that no significant change in fluorescence occured later than 30 minutes after addition of the enzyme. Final fluorescence was calculated as reaction fluorescence minus initial fluorescence;

$$A2 - A1 = A3 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.1$$

On construction of the graph of relative fluorescence (millivolts) versus galactose concentration (mg/l) for the assay of blood samples, the galactose concentration in each standard was multipied by a factor of six in order to correct for the 6 fold dilution which each blood sample underwent on deproteinization with perchloric acid. The relationship between fluorescence and concentration was found to be linear at both the low and high concentration ranges (Figures 5 and 6). The best fit linear regression of fluorescence on galactose concentration was calculated as below,

$$A3 = a + bCg \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.2$$

where Cg = galactose concentration, and the parameters of the line (a and b) were used to calculate the concentration of galactose in blood samples as

described below.

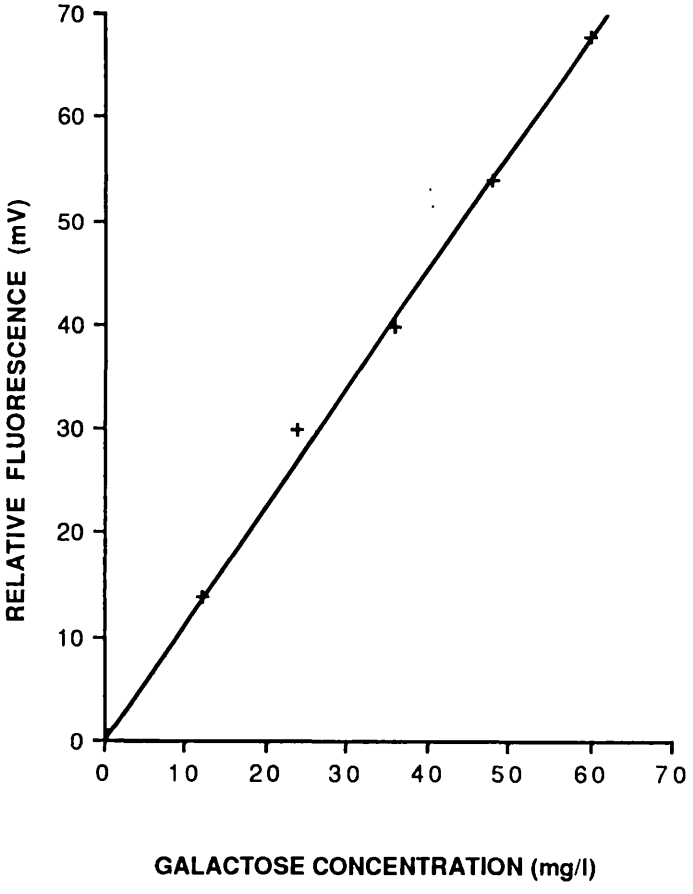


Figure 5

This shows the good correlation between Relative Fluorescence (RF) and Galactose Concentration (GC) in the low galactose concentration range of the calibration graph. The relationship is described by the linear regression equation RF = 1.107GC + 1.286. A correlation coefficient of 0.999 was found and the intercept of the linear regression line with the Y axis was not significantly different from zero.

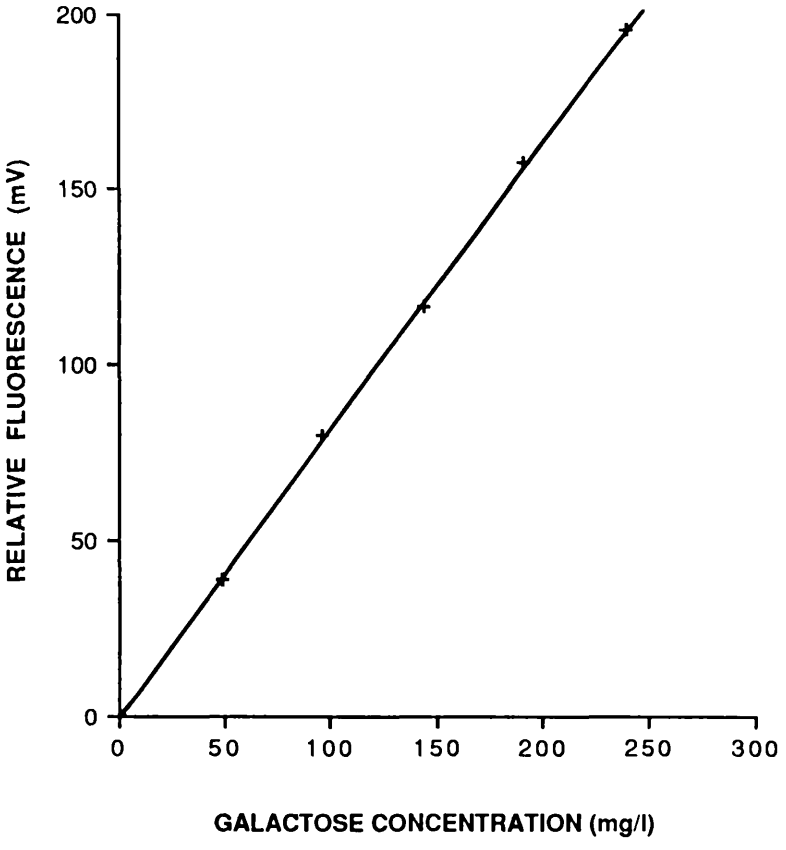


Figure 6

The relationship between Relative Fluorescence (RF) and Galactose Concentration (GC) in the high galactose concentration range of the calibration graph is described by the linear regression equation of RF = 0.8160GC + 2.905. The figure demonstrates the good correlation between relative fluorescence and galactose concentration (correlation coefficient = 0.999). The intercept of the linear regression line with the Y axis was not significantly different from zero.

Sample analysis

Upon analysis 0.2 ml of the deproteinised supernatant from each blood sample was added to 3.0 ml phosphate buffer and 0.10 ml NAD as described above for the calibration standards. The change in fluorescence following addition of 0.02 ml galactose dehydrogenase was similarly determined. Galactose concentration (Cg) was calculated using the parameters a and b derived from equation 3.2 of the calibration curve;

$$Cg = (A3 - a)/b \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 3.3$$

In all experiments, between 3 and 6 blood samples were taken for measurement of basal galactose concentration, which was subtracted from all subsequently measured values. Basal galactose concentration was typically in the range 0 to 5 mg/l.

Urine samples were similarly analysed (results are described in a succeeding chapter), after dilution if necessary to bring galactose concentration into the calibrated range.

ANALYTICAL ACCURACY AND PRECISION

The analytical accuracy and precision of the galactose dehydrogenase assay was assessed by analysing samples prepared by adding known concentrations of galactose to blood collected from fasted rats. Blood samples were obtained from animals anaesthetised with ether and subjected to laparotomy in the following manner; Upon abdominal incision the intestines were expelled from the abdominal cavity and retracted to enable clear visualisation of the inferior vena cava. Blood was withdrawn from the vena cava into a heparinised 10ml syringe. The animal was then sacrificed by opening the chest.

0.9 ml of the collected blood was added to 0.1 ml of a solution of galactose in distilled water at concentrations of 100, 200, 400, 800, 1600 mg/l. These blood standards were treated in the same manner as collected blood samples previously described. Triplicate precipitations were made at each concentration. Six precipitations of the collected blood without adding galactose were made in order to determine basal blood levels of galactose. The average basal galactose concentration measured in each rat was subtracted from the galactose concentration measured in each sample.

After enzyme analysis and determination of galactose

concentration in each sample as already described, the standard deviation between triplicate precipitations was used to assess the precision of the enzyme assay. The accuracy of the enzyme assay was assessed from the measured recovery of galactose from these blood samples.

The same analysis was performed using venous blood obtained from human volunteers after an overnight fast. Again samples were precipitated in triplicate.

Statistical Analysis

In all experiments, the statistical significance between observed differences was tested by the Student's t-test.

RESULTS

The analytical accuracy and precision of the method was assessed from the blood of eight rats and four human volunteers.

The mean measured concentration of galactose from triplicate samples in each rat are tabulated against expected values in Table 1. The overall mean, standard deviation and standard error of the mean for all rats measured for galactose concentration are also shown in Table 1. The mean percentage galactose recovery, at expected galactose concentrations of 10, 20, 40, 80, 160 mg/l was 90.6, 100.15, 99.35, 100.01, and 101.18% respectively (Table 1). No statistically significant difference was found between expected and recovered galactose concentrations.

Simple linear regression analysis demonstrated a correlation coefficient of 0.996 between calculated galactose recovery (CG) and expected concentration of added galactose (EG), with a linear regression equation CG = 1.016EG - 0.847 (Figure 7). The intercept of the linear regression line with the Y-axis was not significantly different from zero, and the gradient was not significantly different from 1.0.

Table 2 demonstrates the standard deviation (SD) and coefficient of variation (CV) between replicate samples at expected galactose concentrations of 10, 20, 40, 80,

160 mg/l. At each concentration, the standard deviation was calculated by pooling the results of triplicate precipitations in eight rats, using the following formula:

$$SD = \sqrt{\frac{\sum_{i=1}^{8}\sum_{j=1}^{3}(CGij - CGi)^2}{(24 - 8)}} \quad \text{.....3.4}$$

where CGij is the calculated galactose recovery for rat i and precipitations j, CGi is the mean of the three precipitations for rat i, and the denominator represents the number of degrees of freedom within groups, i.e. the total number of measurements minus the number of groups (or rats). The coefficient of variation was calculated by expressing SD as a percentage of overall mean CG.

As is common in such assays, standard deviations tend to increase with increasing concentration, however the relationship is not proportional. Depending on concentration, the coefficient of variation (CV) between replicate samples ranged from 4.4% to 17.1% (Table 2). The largest CV's were at the 10 mg/l concentration, and the smallest were at the 80 and 160 mg/l concentration.

The mean measured concentration of galactose from triplicate samples in each human volunteer are tabulated against expected values in Table 3. The

overall mean, standard deviation and standard error of the mean for all volunteers measured for galactose concentration are also shown in Table 3. The mean percentage recovery of galactose, at expected galactose concentration of 10, 20, 40, 80 mg/l was 98.0, 110.0, 105.25, and 106.5% respectively (Table 3). No statistically significant difference was found between expected and recovered galactose concentrations.

Simple linear regression analysis demonstrated a correlation coefficient of 0.992 between calculated galactose recovery (CG) and expected concentration of galactose (EG), with a linear regression equation CG = 1.069EG - 0.284 (Figure 8). The intercept of the linear regression line with the Y-axis was not significantly different from zero, and the gradient was not statistically different from 1.0.

Table 4 shows the standard deviation (SD) and coefficient of variation (CV) between replicate samples at expected galactose concentrations of 10, 20, 40, 80 mg/l. Depending on concentration, the coefficient of variation (CV) between replicate samples ranged from 10.1% to 15.6%. The largest CV's were at the 10 mg/l concentration. Standard deviation values tended to increase with increasing concentration. Values ranging from 1.53 to 8.64 mg/l were found.

TABLE 1

	Expected concentration of galactose (mg/l)				
	10	20	40	80	160
Rat (n = 8)	Mean galactose recovery				
1	6.5	20.1	41.2	82.7	165.7
2	9.6	21.0	41.6	91.1	182.6
3	13.5	22.6	40.9	83.2	162.1
4	9.6	19.9	35.9	80.5	157.0
5	6.6	18.1	37.5	72.9	152.9
6	7.4	23.3	42.6	74.3	149.2
7	7.5	17.7	39.2	80.1	160.6
8	11.8	17.5	39.0	75.3	164.9
MEAN	9.06	20.03	39.74	80.01	161.88
SD	2.55	2.20	2.26	5.93	10.14
SE	0.90	0.78	0.80	2.10	3.59
Mean percent galactose recovery	90.60	100.15	99.35	100.01	101.18

The precision and accuracy of the galactose dehydrogenase enzyme assay has been assessed from triplicate precipitations of blood from eight rats. The individual and overall mean galactose recovery, the standard deviation between animals and the standard error of the overall mean at each expected concentration are tabulated above.

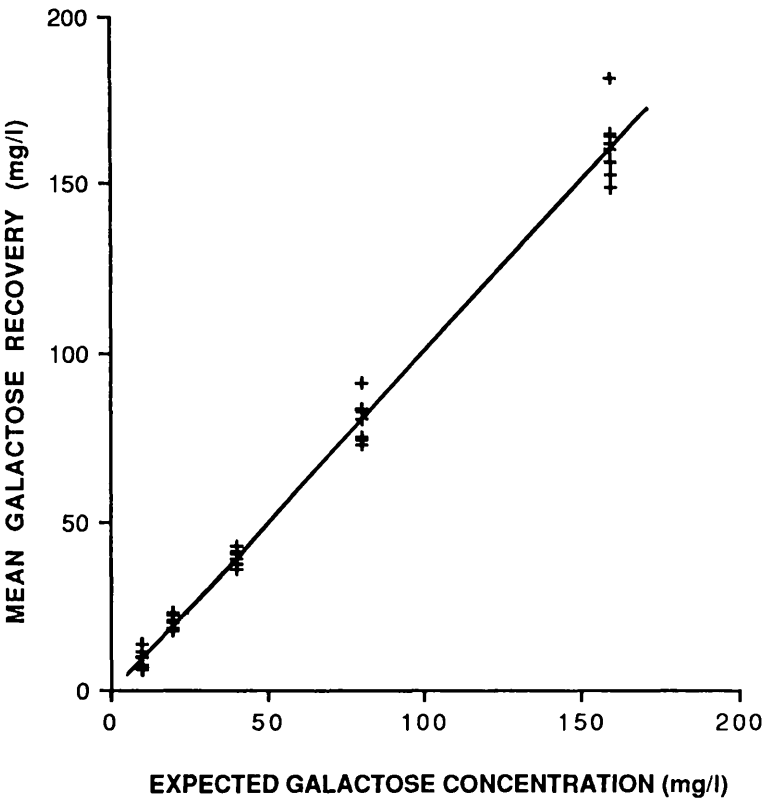


Figure 7

The relationship between Mean Calculated Galactose Recovery (CG) and Expected Galactose Concentration (EG) from eight rats is described by the linear regression equation CG = 1.016EG - 0.847. The correlation coefficient was 0.996. The intercept of the linear regression line with the Y axis was not significantly different from zero, and the gradient was not signficantly different from 1.0.

TABLE 2

	Expected concentration of galactose (mg/l)				
	10	20	40	80	160
SD between replicates (mg/l)	1.55	1.63	2.22	3.96	7.16
CV between replicates (%)	17.1	8.1	5.6	4.9	4.4

Standard deviations and coefficients of variation have been determined between replicate samples measured for galactose concentrations in rats.

TABLE 3

	Expected concentration of galactose (mg/l)			
	10	20	40	80
Human Volunteer	Overall mean of recovered galactose (mg/l)			
SS	10.4	21.8	40.1	83.4
GF	9.1	18.6	39.4	79.7
HB	8.6	23.3	42.2	95.5
DM	11.2	24.3	46.7	82.3
MEAN	9.8	22.0	42.1	85.2
SD	1.19	2.49	3.29	7.02
SE	0.59	1.24	1.64	3.51
Mean percent galactose recovery	98.0	110.0	105.25	106.50

The individual and overall mean galactose recovery, the standard deviation between individuals and the standard error of the mean at each expected concentration are tabulated above.

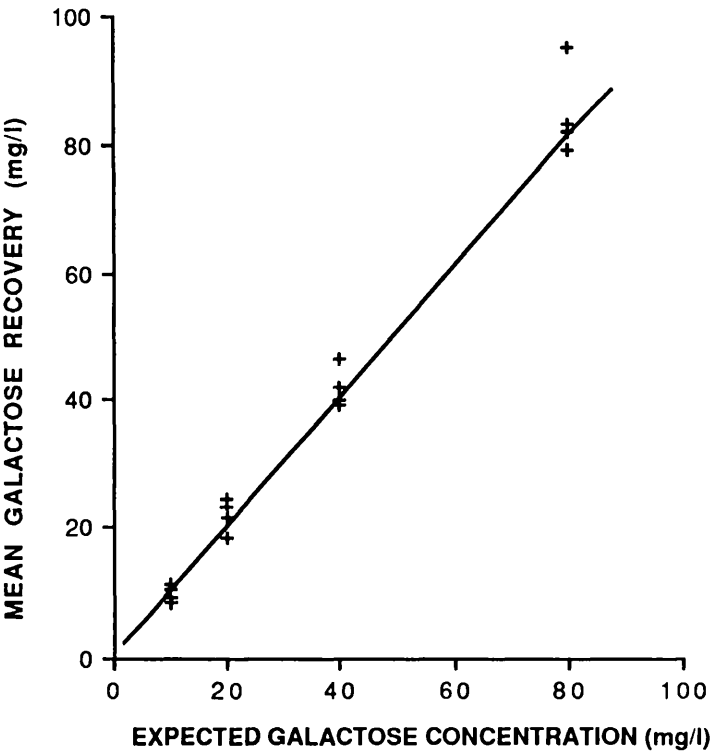


Figure 8

The relationship between Mean Calculated Galactose Recovery (CG) and Expected Concentration of Galactose (EG) from four control volunteers is described by the linear regression equation CG = 1.069EG - 0.284. The correlation coefficient was 0.992. The intercept of the linear regression line with the Y axis was not significantly different from zero, and the gradient was not significantly different from 1.0.

TABLE 4

	Expected concentration of galactose (mg/l)			
	10	20	40	80
SD between replicates (mg/l)	1.53	2.84	5.15	8.64
CV between replicates (%)	15.6	12.9	12.2	10.1

Standard deviations and coefficients of variation have been determined between replicate samples measured for galactose concentrations in human volunteers.

DISCUSSION

The ability to accurately measure galactose concentrations in the blood depends upon the sensitivity and the specificity of the method so applied. Early problems encountered in the analysis of biological solutions have been the presence of other sugars particularly glucose, which react along with galactose, and the inability to measure galactose in physiological concentrations (Raymond et al, 1928; Atkinson et al, 1961). The development of two standard enzyme assays, which are galactose specific, eliminated many of the earlier problems (Henderson and Fales, 1980; Rommel et al, 1968).

The galactose dehydrogenase enzyme assay used in this study was first described by Rommel et al (1968). The specificity of the oxidative enzyme galactose dehydrogenase (EC.1.1.1.48) for galactose was first demonstrated by Wallenfels et al (1962), and early assays were initially coupled to colourimetric reactions (Roth et al, 1965). Sensitivity was enhanced, however, when fluorometry was exploited (Grenier et al, 1973), which proved to be approximately 25-fold more sensitive than the colourimetric galactose oxidase assay as reported by Tengstrom (1969). However, such concentrations which were being measured were still in the concentration range 200 to 600 mg/l.

This study has applied the galactose dehydrogenase enzyme assay with fluorescence spectrophotometry to measure whole blood galactose concentrations at less than 100 mg/l. The assay has been successfully applied to measure galactose concentrations in pigs and humans in excess of 100 mg/l (Keiding et al, 1976; Zaramella et al, 1985). However, few investigators have measured galactose concentrations in the range below 100 mg/l. End point assays, such as this one, should yield the most precise results, being unaffected by small changes in temperature, enzyme activity and time of measurement, since measurement is made after reaction is complete. As is common with such assays, and demonstrated in this study, standard deviations tend to increase with increasing concentration as errors that are constant in percentage terms become more pronounced in absolute terms at higher concentrations. Such concentration-dependent variability is due to random errors in pipetting, sample mixing, concentration preparation and general laboratory techniques. Conversely, coefficients of variation (CV) tend to be increased at low concentrations since measurements at these concentrations are very close to the sensitivity limit of the assay and measurements are often overlaid with instrumental noise. This study demonstrated a CV of 17.1% at an expected concentration of 10 mg/l. A 7.7% CV was reported by Henderson and Fales (1980) at

the same galactose concentration, however, these authors used the alternative assay of galactose oxidase utilising continuous flow fluorometry. This more automated assay would tend to reduce random errors and enhance assay precision. The galactose dehydrogenase enzyme assay has been applied by Keiding (1988) to measure low blood galactose concentrations from human volunteers in hepatic blood flow studies. Arterial galactose concentrations below 18 mg/l and hepatic venous concentrations of almost 0 mg/l have been measured during continuous galactose infusion.

Venous blood from human volunteers in this study demonstrated standard deviations of less than 1.4 mg/l at an expected concentration of 10 mg/l. The standard deviations and coefficients of variation shown in this study are slightly greater than those reported by Henderson and Fales (1980), however they are not unrealistic for this type of assay. At expected concentrations greater than 20 mg/l the scatter of results is small (CV of less than 6%), and the method is reproducible with good resolution. At concentrations of 10 mg/l and below resolution is reduced as one reaches the limits of assay sensitivity. The accuracy of an assay is reflected in the percent recovery of galactose added to blood samples. Henderson and Fales (1980) recovered 97.0%, 99.3%, 101.0%, 98.5% of galactose from blood with expected

galactose concentrations of 10, 40, 70, 100 mg/l. A similar percentage recovery of galactose was achieved from this study at expected galactose concentrations of 10, 20, 40, 80, 160 mg/l. Keiding (1988), however reported lower percent recoveries ranging from 77% to 89% in six human volunteers at a concentration of approximately 20 mg/l.

The accuracy of an assay will owe much to errors which are independent of concentration. Errors which would be a major contributory factor would be those which in some way affected basal galactose measurements. Since the average basal galactose concentration is subtracted from the galactose concentration measured in samples an error producing basal galactose inaccuracies would thus alter final galactose concentration. Fluorescence spectrophotometry is particularly sensitive to contaminating substances and part of the signal detected may in fact not be fluorescence. Raman scattering and Rayleigh scattering, both provide signals to the detector which can be mistaken for fluorescence. Further the cuvette may be contaminated by fluorescent material such as fingerprints. Systematic errors are minimised and accuracy maximised by using linear relations for calibration and by measuring at or near the wavelength of maximal fluorescence, so that a small error in applied wavelength has a minimal effect on the value measured.

The accuracy of the method in this study has proven to be very high and values of recovered galactose are not statistically different from expected galactose concentrations.

CHAPTER FOUR NON-INVASIVE EVALUATION OF PORTASYSTEMIC SHUNTING IN MAN BY D-GALACTOSE AVAILABILITY

INTRODUCTION

SECTION A. ORAL GALACTOSE PILOT STUDY

1. Materials and methods
2. Results
3. Discussion

SECTION B. PATIENT STUDY

1. Materials and methods
2. Results
3. Discussion

INTRODUCTION

Portasystemic shunting is an important consequence of all diseases that lead to portal hypertension. In view of the many abnormalities possibly related to portasystemic shunting it is surprising that so far no non-invasive method has been developed to measure this abnormality in patients and to define its clinical significance.

According to pharmacokinetic principles, as already described in Chapter 2, a procedure for assessing portacaval shunting might be designed by combining oral and intravenous clearances of a substance (Porchet and Bircher, 1982). The naturally occuring monosaccharide D-galactose was found to possess very promising features as a test substance. These features being that galactose is non-toxic, is rapidly and completely absorbed from the gastrointestinal tract, does not undergo intestinal first-pass elimination and is eliminated almost exclusively by the liver by an active metabolic process which has a maximum limiting rate (Vmax). In this chapter galactose was used to evaluate the hypothesis that the systemic availability of oral galactose should indicate the fraction of portal blood by-passing functioning liver hepatocytes, assuming that the systemic spillover mainly depends on intra or extra hepatic shunting.

An initial pilot study was carried out in order to establish a suitable oral dose of galactose, that following absorption from the small intestine was completely cleared from portal blood on its first pass through the liver in normal volunteers who had no history of liver disease. Once established, this dose was given to control patients with no history of liver disease and to patients with liver disease, and a comparison of the systemic availability of the oral galactose dose made. In all patients systemic clearance of galactose was determined following an intravenous infusion of galactose below the maximum elimination rate of the liver (Vmax). The methods used and results are described in this chapter.

Adherance of galactose elimination to first-order kinetics within the concentration range of this study were also examined. If elimination of galactose is a first order process i.e. systemic clearance is independent of concentration, then a doubling of the galactose infusion rate should result in a two fold increase in the equilibrium concentration of galactose. This ideal was examined in control volunteers by calculating galactose clearances from mean systemic equilibrium galactose concentrations at intravenous infusion rates of 40 and 80 mg galactose/min. The ratio of the clearance values from the two infusion rates were then statistically compared to the ideal of

one.

Further, as a test of the method, control volunteers were also given a short intravenous infusion of galactose, and the systemic appearance/clearance curve was analysed as if it had been produced by the same quantity of galactose administered orally. The short intravenous dose thus simulated an oral dose of galactose which was completely absorbed from the small intestine and completely diverted into the systemic circulation. The calculated systemic availability of galactose should thus ideally be 100 percent. The systemic availability was calculated in all subjects utilising both galactose clearance values at intravenous infusion rates of 40 and 80 mg galactose /min. The results obtained were statistically compared to the ideal of 100.

SECTION A. ORAL GALACTOSE PILOT STUDY

MATERIALS AND METHOD

The subjects were all male volunteers with a mean ± SD age of 21.7 ± 1.03 years and a mean ± SD weight of 68.5 ± 5.05 kg. All subjects had fasted for at least nine hours prior to the beginning of the study. All studies were carried out in a quiet room with the subjects supine and their heads elevated to a comfortable angle. The oral dose of galactose ranged from 1.5 to 7 grams, and was dissolved in 50 ml of distilled water. A catheter (Venisystems, Butterfly-23, Abbott Ireland Ltd, Rep.Ireland) was placed in the right forearm vein and maintained by flushing with a dilute heparin solution (Hepsal, CP Pharmaceuticals Ltd, Wrexham). Approximately 0.5 ml blood samples were collected at 2 to 4 minute intervals for a total of 40 minutes following administration of the oral dose. Prior to all sample collections a 1 ml blood sample was collected and discarded. This sample contained largely heparinised saline which maintained the venous line. 0.1 ml of the collected blood was added to 0.5 ml perchloric acid, centrifuged and the supernatant measured for galactose concentration as described in Chapter 3. Prior to administering the oral dose a venous blood sample was collected to allow

determination of basal galactose concentrations.

In only two subjects were systemic clearance measurements made. In these volunteers galactose dissolved in distilled water was infused into an indwelling catheter (Venisystems, Butterfly-23, Abbott Ireland Ltd, Rep.Ireland) in a left forearm vein by a Treonic IP3 infusion pump (Vickers Medical, Basingstoke, England) at a rate of 30 ml/hr (40 mg galactose/min). Blood samples were collected at 10 minute intervals from 50 to 90 minutes after the start of the infusion. The oral and clearance studies were performed on different dates, and the systemic availability calculation in these two subjects was based on the assumption that clearance had a similar value on both occasions. Systemic availability of the oral dose in the two subjects was calculated as described below.

AUC, clearance and systemic availability calculations

In all subjects, areas under the galactose appearance curve (AUC) from concentration time graphs were determined by a specially written computer program, using linear interpolation between points.

In two subjects systemic galactose clearance (K) was calculated using equation 2.14 in chapter 2,

$$I/Ca^{iv} = K \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.14)$$

where, I = rate of galactose infusion
Ca^{iv} = mean equilibrium blood galactose concentration following an intravenous infusion

The mean equilibrium venous concentration of galactose (Ca^{iv}) was determined by subtracting the basal concentration of galactose at time zero (Co) from the mean equilibrium galactose concentration (Css) i.e. $Ca^{iv} = Css - Co$.

The systemic availability of galactose was calculated using equation 2.15 in chapter 2, where

$$SA = I.AUC/Q.Ca^{iv} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2.15)$$

Where, SA = Systemic availability of galactose
AUC = area under the galactose appearance curve
I = rate of galactose infusion
Q = oral galactose dose
Ca^{iv} = mean equilibrium venous blood galactose concentration

RESULTS

A clearly defined blood galactose appearance peak was only seen in subjects who received at least 5 grams of galactose orally (Table 5). Blood galactose reached a maximum level 14 to 18 minutes after administration of the oral dose, and had declined to basal level by 40 minutes in all but one subject, who received 7 grams of galactose and showed peak and 40 minute concentrations of 46 mg/l and 6 mg/l respectively. In subjects who received 3 grams of galactose or less, and in one out of two who received 5 grams, there was no well-defined appearance peak, and the maximum concentration recorded in blood did not exceed 4 mg/l above baseline.

In the two subjects in whom clearance measurements were performed, blood galactose concentration was constant over the 40 minute sampling period to within 2 mg/l, and the average concentration was used for calculation. Clearance values of 2.58 l/min and 1.78 l/min were obtained, giving estimates of 4.3% and 17.4% for the systemic availability of oral doses of 3 grams and 7 grams of galactose respectively (Table 5).

TABLE 5

Subject	Dose (g)	CGmax (mg/l)	Tpk (min)	AUC (mg.min/l)	Clearance (l/min)	SA (%)
1	1.5	3	a	30	----	--
2	3.0	1	a	16	----	--
3	3.0	4	a	47	----	--
4	5.0	4	a	50	2.58	4.3
5	5.0	16	18	115	----	--
6	7.0	18	14	186	----	--
7	7.0	46	18	687	1.78	17.4

CGmax = maximum blood galactose concentration after oral dose
Tpk = time of blood galactose appearance peak
a = no clearly defined peak observed
AUC = area under appearance curve from 0 to 40 minutes
Clearance = I/Ca^{iv}
SA = Systemic availability of oral dose (eg.I.AUC/oral dose.Ca^{iv})

This shows the area under the galactose appearance curve and other calculated expressions at various oral doses of galactose in control volunteers.

DISCUSSION

The aim of this pilot study was to determine an oral dose of galactose that after absorption from the small intestine would be completely cleared by the liver in control volunteers. The results show that oral doses of 5 grams of galactose and above are not completely extracted on first pass through the normal liver, although the calculated systemic availability of a 7 gram dose in the subject showing the highest level of breakthrough was still less then 20%. The ability to detect an apperance peak at lower doses is limited by measurement accuracy, and determination of the area under the appearance curve is subject to errors of measurement, particularly in the basal level of blood galactose which is the reference point for all subsequent measurements. An error of 1 mg/l in this basal level would lead to an error of 40 mg.min/l in the area under the appearance curve. However, in absolute terms this would translate into only a small error in systemic availability. Such errors would be minimised by choosing as large a dose as is consistent with high extraction efficiency by the normal liver, and on the basis of this pilot study it was decided to use a dose of 4 grams for further investigations. It was also decided that blood sampling following the oral dose shuld be extended to 60 minutes, and should be

most frequent during the first twenty minutes to cover the relatively steep rise to the concentration peak.

SECTION B. PATIENT STUDY

MATERIALS AND METHODS

SUBJECTS

A). Control volunteers

Six control volunteers from our Department of Surgery, Glasgow Royal Infirmary, were studied for the purpose of assessing the adherance of galactose elimination to first-order kinetics. The same volunteers were also studied for the purpose of assessing whether or not a short intravenous infusion of galactose, treated as a dummy oral dose, which ideally should produce a systemic availability of 100 percent, did indeed produce this ideal. The 5 men and 1 woman studied had a mean age of 31.6 $\pm$ 7.02 (SD) years and had a weight range of 55.0 to 85.7 kg. All were healthy and had normal biochemical data (Table 6).

B). Patients with and without liver disease/portal hypertension

Six control subjects without, and seven subjects with liver disease and one patient with portal vein thrombosis were studied over a 2 hour time period in order to examine the systemic availability of galactose

following a low oral dose of galactose. All six subjects without liver disease were studied exclusively for the purpose of establishing normal data. The 5 men and 1 woman had a mean age of 65.8 ± 8.5 (SD) years, and ranged in weight from 58.8 to 92.0 kg. All were healthy and had normal values of total bilirubin, aspartate transaminase and alanine transaminase, gamma-glutamyl transpeptidase, alkaline phosphatase (Table 7). In all cases the contol patients were in a pre-operative state and had been admitted to the Glasgow Royal Infirmary for minor operations, for example, surgery for a lipoma on the arm and removal of gallstones.

The seven patients with liver disease and one patient with portal vein thrombosis had a mean age of 51.2 ± 11.96 (SD) years and had been referred to our department by practicing physicians. The weights of the patients ranged from 52.0 to 90.0 kg. All patients had evidence of portal hypertension with portasystemic shunting in that they had endoscopically-diagnosed oesophageal varices. All but two patients had elevated fasting bile acid concentrations. The patients are defined in Table 7 by conventional biochemical data.

TABLE 6

Volunteers	Albumin (g/l)	Bilirubin (umol/l)	AlkPhos (U/l)	AST (U/l)	ALT	Gamma GT (U/l)	Glucose (fasting) (mmol/l)
JA	47	13	205	17	9	47	5.1
GF	45	7	115	11	8	82	4.9
DMcM	44	17	140	20	11	15	5.3
WA	45	11	120	10	<5	13	5.7
HB	46	5	100	15	<5	6	4.9
HT	41	7	75	10	12	9	5.1
Normal Range	35-55	3-22	80-280	12-48	3-55	<36	4-5.5

Alk Phos = Alkaline Phosphatase
AST = Aspartate Transaminase
ALT = Alanine Transaminase
Gamma GT = Gamma-glutamyl Transpeptidase

This shows the biochemical data for six control volunteers. Normal ranges are given as defined by the Biochemistry Department, Glasgow Royal Infirmary. All had normal biochemical data, except volunteers JA and GF who had slightly elevated Gamma GT levels above normal.

TABLE 7

Patients	Albumin (g/l)	Bilirubin (umol/l)	AlkPhos (U/l)	AsT (U/l)	ALT	Gamma GT (U/l)	Glucose (fasting) (mmol/l)
Without liver disease/portal hypertension							
JC	45	9	135	20	13	38	5.1
FMcC	47	10		16	<5	25	6.0
RS	46	23	155	23	8	41	7.3
JA	48	10	160	23	7	19	5.4
CMcN	32	10	140	<5	<5	9	6.0
RH	--	4	210	20	23	35	---
With liver disease/portal hypertension							
TM	41	25	275	22	6	206	8.6
GL	26	120	---	71	21	191	5.1
AMcM	34	32	320	42	<5	287	6.1
WMcC	29	19	160	24	<5	93	5.9
JN	30	44	430	40	8	76	5.8
*AMcA	40	26	250	30	9	32	5.6
JE	34	11	980	25	33	340	---
MK	30	34	1320	82	27	147	---
Normal Range	35-55	3-22	80-280	12-48	3-55	<36	4-5.5

* = patient with portal vein thrombosis

This shows the biochemical data, for patients with and without liver disease/portal hypertension. Normal ranges are given as defined by the Biochemistry Department, Glasgow Royal Infirmary.

The definitions of the enzymes in Table 7 are shown below;

Alk Phos = Alkaline Phosphatase
AST = Aspartate Transaminase
ALT = Alanine Transaminase
Gamma GT = Gamma-glutamyl Transpeptidase

METHOD

A) Control volunteers

All control volunteers were asked to fast overnight and throughout the study. All studies were carried out in a quiet room. Studies were carried out with the subjects supine and their heads elevated to a comfortable angle. A solution of eight percent D-galactose (BDH Chemicals Ltd, Poole) was prepared by the Pharmacy Department, Glasgow Royal Infirmary, and was given by intravenous infusion into the left forearm vein catheter (Venisystems, Butterfly-23, Abbott Ireland Ltd, Rep.Ireland) at a constant rate of 90 ml/hr by Treonic IP3 infusion pump (Vickers Medical, Basingstoke, England) for 30 minutes. 1 ml blood samples were collected, from an indwelling catheter (Venisystems, Butterfly-23 Abbott Ireland Ltd, Rep.Ireland) in the right forearm vein, throughout this time period approximately every 4 minutes, and sampling was then continued after the end of the infusion until 60 minutes had elapsed. The venous catheter was maintained by flushing with a dilute heparin solution (Hepsal, CP Pharmaceuticals Ltd, Wrexham). Data from this part of the experiment were subsequently analysed as if the 3.6 grams of galactose infused over 30 minutes had in fact been given orally. Prior to beginning the 90 ml/hr infusion a blood sample was

collected to allow determination of basal galactose concentrations.

A second intravenous infusion at a rate of 30 ml/hr (40 mg galactose/min) was performed thereafter for an hour followed by a third infusion at 60 ml/hr (80 mg galactose/min) for a further hour (Treonic IP3 infusion pump). No priming doses were used in any of the infusions. In both the latter infusions 1 ml blood samples were collected every five minutes after equilibrium had been reached, approximately 30 minutes after beginning the infusion, and continued thereafter until 60 minutes.

In all cases 0.1 ml of the collected blood sample was immediately added to 0.5 ml, 0.33 mol/l perchloric acid and microcentrifuged for three minutes, as described in Chapter 3. Double precipitations of each sample were made, and samples analysed as described in Chapter 3.

Prior to all sample collections an initial 1 ml blood sample was drawn and discarded. This sample contained largely heparinised saline which maintained the venous line.

Area under the galactose appearance curve from the first infusion, galactose clearance at the subsequent two infusion rates, and systemic availability were calculated as described in Section A.

B) Patients with and without liver disease

Patients were similarly prepared as described above. All patients were fasted overnight and throughout the study. 50 ml of a solution of eight percent D-galactose, prepared by the Pharmacy Department, Glasgow Royal Infirmary, was given orally to the patients. 1 ml blood samples were collected from an indwelling catheter in the right forearm vein (Venisystems, Butterfly-23, Abbott Ireland Ltd, Rep. Ireland), every 2 minutes for the first 20 minutes and thereafter every four minutes until 60 minutes. Prior to administering the oral dose a blood sample was collected to allow determination of basal galactose concentrations. Upon completion of oral blood sampling an intravenous infusion of galactose was given in order to determine systemic clearance of galactose. A constant intravenous infusion rate of 30 ml/hr (40 mg galactose/min) was given by Treonic IP3 infusion pump (Vickers Medical, Basingstoke, England) into the left forearm vein catheter (Venisystems, Butterfly-23, Abbott Ireland Ltd, Rep.Ireland). 1 ml blood samples were collected every five minutes, after equilibrium had been reached, approximately 30 minutes after beginning the infusion, and continued thereafter until 60 minutes. 0.1 ml of each collected blood sample was deproteinised and analysed as described in Chapter 3.

Again double precipitations of each sample were made. Prior to all sample collections an initial 1 ml blood sample was drawn and discarded. This sample contained largely heparinised saline which maintained the venous line.

The area under the galactose appearance curve, galactose clearance at an infusion rate of 40 mg galactose/min and systemic availability were calculated as described in Section A.

Statistical analysis

In control volunteers the mean ratio of the calculated galactose clearance values at intravenous infusion rates of 40 and 80 mg galactose/min, which ideally should equal one if elimination of galactose in the concentration range chosen in this study is first-order, was tested equal to one by the Student's t-test. Similarly, in the same control volunteers, the systemic availability of a short intravenous infusion, at a rate of 90 ml/hr, which ideally should result in a bioavailability of galactose of 100 percent, was tested equal to 100 by the Student's t-test.

In control and liver diseased patients the statistical significance between observed differences in was tested by using the Student's t-test.

RESULTS

CONTROL VOLUNTEERS

All subjects achieved equilibrium whole blood galactose concentrations at intravenous galactose infusion rates of 40 and 80 mg galactose/min between 30 and 60 minutes after beginning the infusion. Figure 9 shows systemic whole blood equilibrium galactose concentrations in a control volunteer at infusion rates of 40 and 80 mg galactose/min. Systemic galactose concentrations were constant to within 2 mg/l, and the average concentration was used for calculation of systemic galactose clearance.

Individual clearance values in six control volunteers at an intravenous galactose infusion rate of 40 and 80 mg galactose/min ranged from 1320 to 2303 ml/min and 1670 to 1970 ml/min respectively (Table 8). The mean galactose clearance $\pm$ SD was 1851 $\pm$ 330.6 ml/min and 1812.5 $\pm$ 126.1 ml/min at intravenous infusion rates of 40 and 80 mg galactose/min respectively (Table 8). Galactose clearance adhered to first-order kinetics within the concentration range of this study. The ideal, a doubling of the systemic whole blood galactose concentration with a doubling of the infusion rate, was approached in all volunteers. In only four out of the six control volunteers originally studied was galactose clearance actually measured at the higher intravenous

infusion rate of 80 mg galactose/min. The was due to a pump failure in one study and a power cut in the other. The individual ratio of clearance at a galactose infusion rate of 80 mg galactose/min divided by clearance at a galactose infusion rate of 40 mg galactose/min in four control volunteers ranged from 0.903 to 1.027, with a mean ± SD of 0.970 ± 0.053. This mean ratio was not significantly different from the ideal of 1.00 ($p = 0.172$) (Table 9).

Table 8 also shows individual areas under the galactose appearance curve after a 90 ml/hr short intravenous infusion of galactose, and the corresponding systemic availability of galactose expressed as a percentage and calculated using clearance values at infusion rates of 40 and 80 mg galactose/min in control volunteers. A mean ± SD area under the galactose appearance curve (AUC) of 2291.3 ± 389.7 mg.min/l was found after a 30 minute intravenous infusion at a rate of 90 ml/hr in control volunteers. Individual values for AUC ranged from 1916 to 2963 mg.min/l (Table 8). Figure 10 shows the typical form of the galactose concentration time curve following the short intravenous infusion of galactose at 90 ml/hr in a control volunteer.

The ideal of simulating the complete absorption of an oral dose of galactose which resulted in a systemic availability of galactose of 100 percent by infusing

intravenously galactose at a rate of 90 ml/hr, was approached in all control volunteers. Individual systemic availability values calculated using galactose clearance values at intravenous infusion rates of 40 and 80 mg galactose/min, ranged from 99.0 to 122.6 percent and 99.5 to 105.7 percent respectively (Table 8). The mean ± SD systemic availability of galactose was 108.7 ± 9.2 percent and 102.5 ± 2.6 percent at clearance values determined at intravenous infusion rates of 40 and 80 mg galactose/min respectively (Table 8). Neither of these mean shunting values were significantly different from the ideal of 100 ($p > 0.01$).

PATIENTS WITH AND WITHOUT LIVER DISEASE

All patients achieved equilibrium whole blood galactose concentrations at a galactose intravenous infusion rate of 40 mg galactose/min between 30 and 60 minutes after beginning the infusion, as already shown in Figure 9 for control volunteers.

Individual systemic galactose clearance values, areas under the galactose appearance curve and percent systemic availability for control patients without liver disease and patients with liver disease are tabulated in Table 10.

Systemic galactose clearance ranged from 1064 to 1835

ml/min and 892 to 2060 ml/min in control patients and patients with liver disease at a intravenous infusion rate of 40 mg galactose/min respectively (Table 10). The mean systemic galactose clearance ± SD for control patients without liver disease was 1455.7 ± 288.49 ml/min which was not significantly different from patients with liver disease, who showed a mean ± SD of 1482.6 ± 375.46 ml/min at a galactose infusion rate of 40 mg galactose/min (Table 10). Neither the mean galactose clearance in control patients and patients with liver disease was significantly different from the mean galactose clearance found in control volunteers, 1851 ± 330.6 ml/min, at an intravenous infusion rate of 40 mg galactose/min.

The area under the galactose appearace curve (AUC) following oral ingestion of 4g of galactose in control patients without liver disease ranged from 177 to 519 mg.min/l, with a mean ± SD of 351.8 ± 130.5 mg.min/l. The AUC in control patients was significantly lower than the area under the galactose appearance curve in patients with liver disease, who showed AUC to range from 799 to 3185 mg.min/l, with a mean ± SD of 1940.1 ± 863.35 mg.min/l (Table 10). Figures 11 and 12 show the the differing areas under the galactose appearance curve after a 50 ml (4 gram) oral dose of galactose in a control patient and a patient with liver disease.

Individual systemic availability of galactose in

control patients and patients with liver disease ranged from 7.1 to 23.8 percent and 26.4 to 108.0 percent respectively (Table 10). The mean $\pm$ SD systemic availability in patients with liver disease was 72.19 $\pm$ 33.38 percent which was significantly different ($p < 0.005$) from the systemic availability found in control patients without liver disease who showed a mean $\pm$ SD of 13.65 $\pm$ 6.31 percent (Table 10).

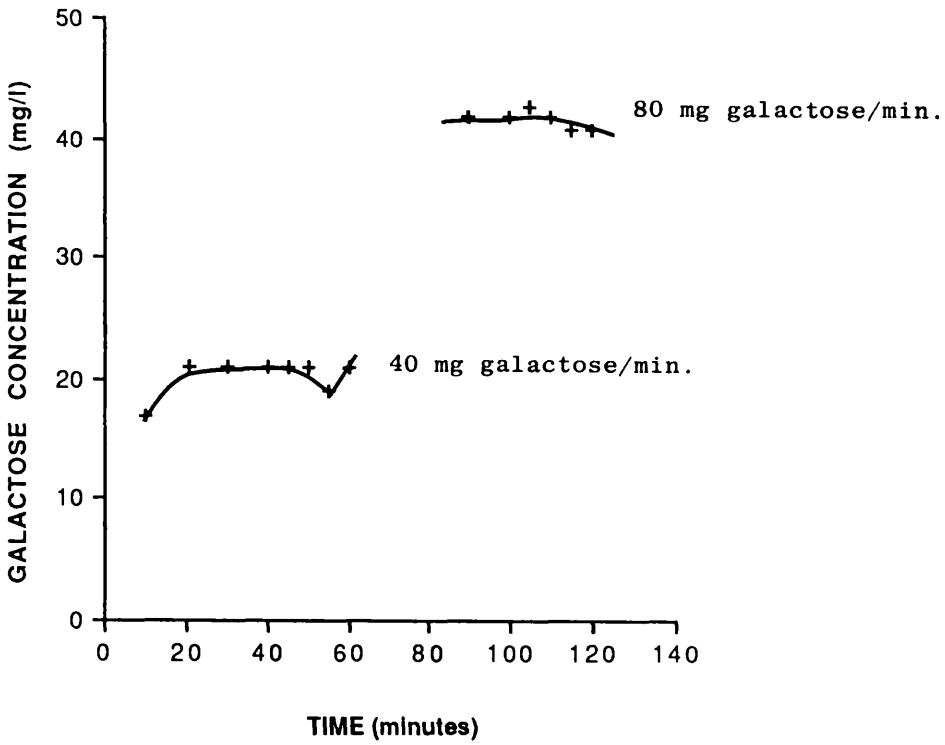


Figure 9

This shows whole blood equilibrium galactose concentrations in a control volunteer at intravenous infusion rates of 40 and 80mg galactose/min. If elimination of galactose by the liver follows first order kinetics then a doubling of the intravenous infusion rate will result in a doubling of systemic galactose concentration.

TABLE 8

Control Volunteers	Clearance (ml/min)		AUC (mg.min/l)	Systemic availability (%)	
	40 (mg/min)	80 (mg/min)		40 (mg/min)	80 (mg/min)
WA	1850	1670	2283	117.4	105.7
GF	2060	1970	2020	104.1	99.5
HT	1320	----	2963	105.1	----
JA	1850	1840	2499	104.2	103.3
HB	1723	1770	2067	99.0	101.6
DM	2303	----	1916	122.6	----
Mean	1851	1812.5	2291.3	108.7	102.5
SD	330.6	126.1	389.7	9.2	2.6

Clearance = Volume of blood completely cleared of galactose by the liver per minute
AUC = Area under the galactose appearance curve following a short intravenous infusion

This shows whole blood galactose clearance at intravenous infusion rates of 40 and 80 mg galactose/min, the area under the galactose appearance curve calculated from a 30 minute intravenous infusion at a rate of 90 ml/hr, and the systemic availability of galactose at both clearance values in control volunteers.

TABLE 9

	CONTROL VOLUNTEERS			
	WA	GF	JA	HB
Infusion rate 1 (mg galactose/min)	40	40	40	40
Infusion rate 2 (mg galactose/min)	80	80	80	80
Clearance 1 (ml/min)	1850	2060	1850	1723
Clearance 2 (ml/min)	1670	1970	1840	1770
Clearance 2 / Clearance 1	0.903	0.956	0.995	1.027

This table demonstrates galactose clearance measured at two infusion rates (Infusion 2 = Infusion 1 x 2) in each control volunteer on the same day. The mean $\pm$ SD ratio clearance 2/ clearance 1 was 0.970 $\pm$ 0.053. This ratio was not significantly different from the ideal of 1.00.

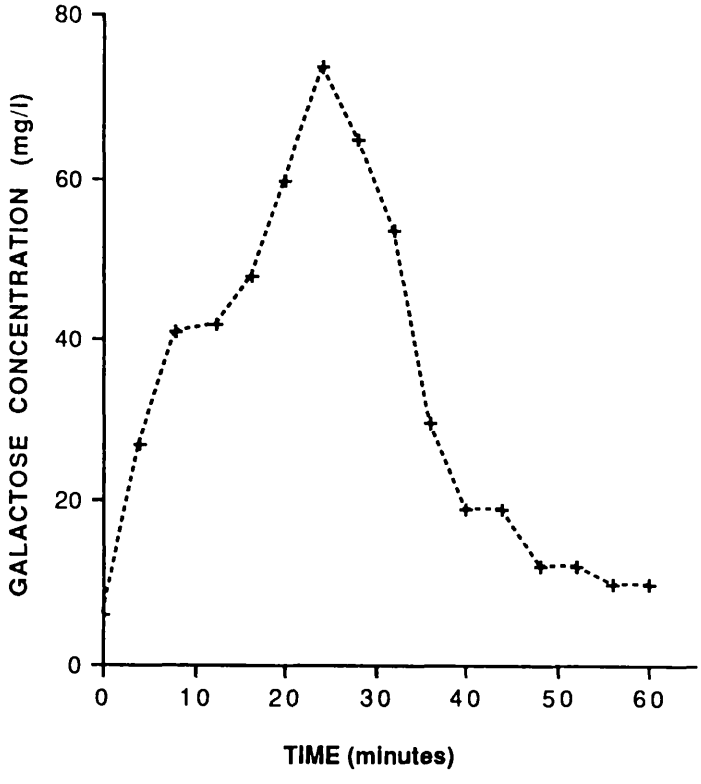


Figure 10

This shows the galactose concentration time curve following a 30 minute intravenous infusion of galactose at a rate of 90 ml/hr in a control volunteer. The area under the galactose appearance curve was 2020 mg. min/l.

TABLE 10

Subject	Clearance 40 mg/min (ml/min)	AUC (mg.min/l)	Systemic availability (%)
CONTROLS			
RS	1780	375	16.8
AMcC	1064	458	12.2
JA	1230	230	7.1
CMcN	1403	352	14.7
JC	1835	519	23.8
RH	1494	177	7.3
Mean	1455.70	351.80	13.65
SD	288.49	130.50	6.31
PATIENTS			
WMcC	2060	1866	96.2
*AMcA	1754	983	43.1
TM	892	1727	38.5
GMcM	1794	2265	101.6
JN	1490	1655	61.7
GL	1276	3185	102.0
JE	1191	799	26.4
MK	1404	3041	108.0
Mean	1482.60	1940.10	72.19
SD	375.46	863.35	33.38

* = Patient with portal vein thrombosis

Whole blood galactose clearance at an intravenous infusion rate of 40 mg galactose/min, the areas under the galactose appearance curve calculated after a 50

ml (4 gram) oral dose of galactose, and the systemic availability of galactose are shown in control patients and patients liver disease/portal hypertension.

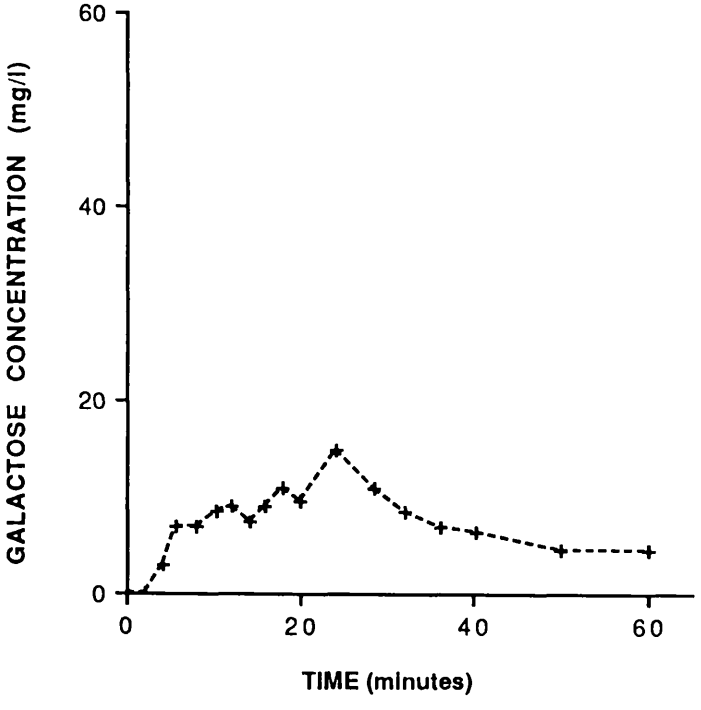


Figure 11

This shows the galactose concentration time curve after oral administration of 4 grams of galactose to a control patient. The area under the galactose appearance curve was 230mg min/l and percent systemic availability was 7.1.

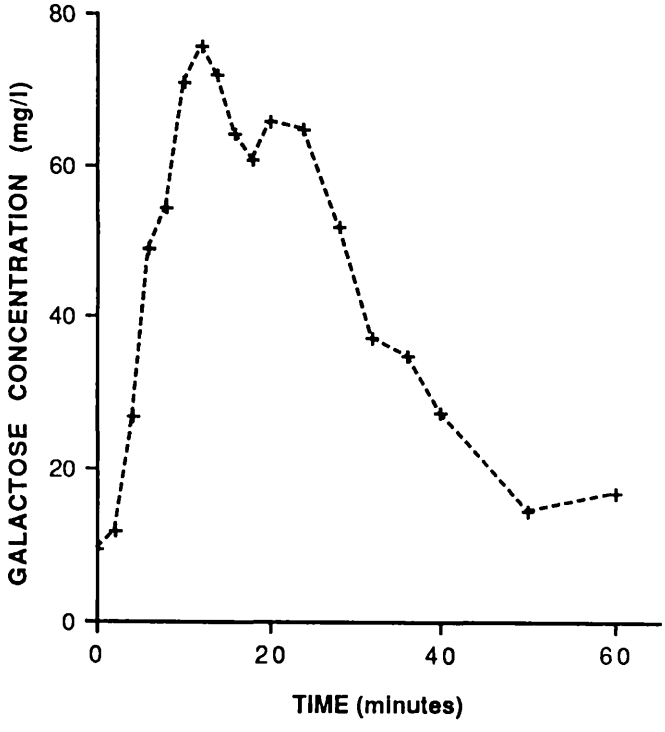


Figure 12

This shows the galactose concentration time curve after oral administration of 4 grams of galactose to a patient with liver disease and portal hypertension. The area under the galactose appearance curve was 1866mg min/l and percent systemic availability was 96.2.

DISCUSSION

The majority of methods employed to quantitate portasystemic shunting are invasive in nature, requiring intrasplenic injection of radioactive indicator substance and hepatic venous sampling (Caesar et al, 1962; Groszmann et al, 1972). Only within the last ten years have realistic non-invasive attempts been made to quantitate the magnitude of portasystemic shunting. The basis of work carried out in this thesis arises from research by Porchet and Bircher (1982) in which a procedure for assessing portasystemic shunting was designed which combined oral and intravenous clearances of a susbstance. The aim of their study and indeed this study was to administer sufficiently low oral doses of a substance which on first pass through the normal liver would be completely extracted. The same oral dose when administered to a subject with liver disease/portal hypertension would by-pass the liver in proportion to the degree of portasystemic shunting, and the systemic availability of the oral dose would thus be a measure of the magnitude of portasystemic shunting.

The data of this study suggest that, in patients with portal hypertension and/or liver disease galactose may be a valuable test compound for the non-invasive assessment of portasystemic shunting. The procedure is

safe, simple, well accepted by patients and the inconvenience appeared to be minimal. Furthermore, the subjects investigated had to remain within the investigation laboratory for only two hours. These conclusions, however, have to be carefully reviewed within the light of the limitations imposed by the chosen methodology.

On theoretical grounds, a clearance test which is designed for the investigation of portasystemic shunting should fulfill the following requirements: the test compound should be non-toxic and easy to measure; it should be absorbed completely and rapidly from the gastrointestinal tract; intestinal first-pass elimination should be negligible; once in the portal vein, it should be removed virtually completely in a single passage through the normal liver, i.e., through a normal sinusoidal pathway from the portal triad to the central vein; the fraction of the test compound reaching the systemic circulation should be cleared by reproducible first-order mechanisms rendering an intravenous and oral comparison valid.

Galactose fulfills the above postulates to a high degree. Galactose is a naturally occuring monosaccharide and is non-toxic, thus oral and intravenous doses of galactose are safe. Whole blood concentrations of galactose between 0 and 100 mg/l can be measured specifically and easily by the galactose

dehydrogenase enzyme assay, as described in chapter 3, (Wallenfels et al, 1962). The accuracy of measuring galactose concentrations below 10 mg/l tends to decrease as we approach the limits of assay sensitivity.

Absorption of 4 grams of galactose from the gastrointestinal tract is rapid as evidenced by raised blood concentrations of galactose within 10 minutes after an oral dose and a concentration peak at about twenty minutes.

To our knowledge galactose does not undergo metabolised in the duodenal mucosa while being absorbed from the gastrointestinal tract.

The liver is the major organ for galactose elimination, wherein several known enzymatic processes convert it to glucose through the sugar nucleotide pathway (Kalckar, 1957). Extraction of galactose by the liver is also very high in normal subjects, between 93 and 95 percent (Henderson et al, 1982). The short half life of galactose of approximately 10 to 30 minutes (Henderson et al, 1982), is an important advantage, because it allows establishment of a steady-state within a few minutes, thus ensuring in this study that hepatic clearance of galactose is flow dependent , and that bioavailability of orally administered doses reflects portacaval shunting only. Extrahepatic elimination of galactose, for example urinary excretion, renal

metabolism and erythrocyte metabolism does not appear to be quantitatively significant. In a study by Henderson et al (1982) urinary excretion and erythrocyte metabolism accounted for only 4 percent of the galactose eliminated.

The technique for assessing galactose systemic availability requires the assumption of first-order kinetics. Saturation of first-pass elimination of galactose is well recognised, (Colcher et al 1946; Tygstrup, 1964). Therefore, in a study such as this it was necessary to apply small oral doses of 4 grams of galactose which were almost completely eliminated in control volunteers, as shown in section A of this chapter. Such small oral doses avoided any evidence of hepatic saturation. Evaluation of the non-steady state kinetics after the oral dose with steady-state (equilibrium) kinetics after the intravenous infusion appears, therefore, to be justifiable. Empirically this approach has been verified by the rapidly increasing and decreasing systemic galactose concentrations at an intravenous infusion rate of 90 ml/hr in control volunteers which simulated the non-steady state of an oral dose but assured a 100 percent systemic availability. The result of 108.7 and 102.5 percent systemic availability of galactose calculated by using systemic clearance values at steady-state intravenous infusion rates of 40 and 80 mg galactose/hr, confirms

this general procedure.

In all subjects in this study systemic galactose clearance was calculated from mean equilibrium galactose concentrations, during a continuous intravenous infusion at a rate well below the intrinsic elimination capacity of the liver in a deliberate attempt to study flow-dependent clearance. Tygstrup (1964) found the galactose elimination capacity in control subjects to be 440 mg galactose/min and in patients with alcoholic cirrhosis to be 263 mg galactose/min. In this study the intravenous infusions of galactose were performed at infusion rates of 40 and 80 mg galactose/min. Reproducible first-order kinetics have been well documented for galactose (Tygstrup and Winkler, 1958; Henderson et al, 1982). In this study the ratio of clearance at an intravenous infusion rate of 40 mg galactose/min to clearance at an intravenous infusion rate of 80 mg galactose/min in control volunteers did not vary significantly from the ideal of one. This data shows galactose elimination to be first-order within the defined ranges in this study, and the high extraction of galactose by the liver is maintained when the infusion rate is doubled. A similar study carried out by Henderson et al (1982) found that a doubling of the infusion rate from 50 to 100 mg galactose/min reduced systemic galactose clearance by 8.1 percent. This fall was attributed to a

combination of a reduction in hepatic extraction, the effect of extrahepatic elimination, and the possible influence of non-free galactose derivatives such as amino sugars and galactosides on the clearance calculation. It is possible that the absence of any such effect in the present study is due to the lower rates of galactose infusion, which would be less likely to give rise to a departure from first-order kinetics. In agreement with the earlier postulates, the bioavailability of a 4 gram oral dose of galactose was acceptably low in control patients with no history of liver disease, but was nonetheless higher than expected on the basis of the pilot study in young healthy volunteers. This may have occured due to the age difference and general health difference between the two groups. However, in patients with documented portasystemic shunting, as assessed by independent methods, the same oral dose produced much greater areas under the curve and systemic availabilities ranged from 26.4 to 108.0 percent. To our knowledge no investigators have previously used galactose as a test compound to assess the magnitude of portasystemic shunting in patients with liver disease. In a recent study carried out by Cavanna et al (1987) the bioavailability of the naturally occuring polyol D-sorbitol was used to assess the magnitude of portasystemic shunting in a group of control and liver

diseased patients. In normal patients the bioavailability of sorbitol was found to be zero and 29 percent in cirrhotic patients. However, in patients with portacaval shunts an expected bioavailability of 100 percent was reduced to 38 percent, due to intestinal first-pass elimination of at least 50 percent of the oral sorbitol dose. Another method using glyceryl trinitrate, and based on the same principle as this study, did almost give 100 percent bioavailability in patients with portacaval shunts (Porchet and Bircher, 1982). Porchet and Bircher's technique was, however, assessed in the University Department of Surgery at Glasgow Royal Infirmary (Garden and Angerson, unpublished observations) and it was found that the indirect plethsmographic technique used to measure circulating nitroglycerine was subject to large errors. It would thus appear to be very difficult to find test substances which are not subject to some form of measurement difficulity and/or suffer from intestinal first-pass elimination.

From the results of this study, it would appear, that galactose is by far a better test compound when applied orally to assess portasystemic shunting in liver diseased patients. Although in the present study there was no patient who was independently known to have 100 percent shunting, the fact that values close to 100

percent were found in four cirrhotic patients suggests that there is unlikely to be significant underestimation of portasystemic shunting because of underestimated elimination of galactose.

The slight systemic availability of galactose seen in the six control patients may be due to galactose absorption through oral or oesophageal mucosa which may bypass the liver and simulate portasystemic shunting. It was assumed that out of the total volume of 50 ml of galactose only a few milliliters might be retained above the diaphragm for galactose absorption. Correspondingly, the error should be small. Another possible source of error, which would underestimate the availability of an oral dose of galactose, arises if galactose is infused intravenously. Here some of the sugar may be lost in the tubing of the infusion set, thereby leading to a systematic error in the calculation of infusion rates and systemic availabilities.

The galactose test, however, seems to provide information that hitherto has been available only through highly invasive (Shand, 1979; Okuda et al, 1977) or time consuming (McLean et al, 1979) investigations. Inasmuch as incomplete clearance of a high extraction substance from portal blood represents portasystemic shunting, the galactose test described in this study may be regarded as a tool to assess this

abnormality.

CHAPTER FIVE BASIC ANIMAL METHODS

INTRODUCTION

SECTION A. BASIC MODEL

1. Anatomy of the rat

SECTION B. A PREHEPATIC MODEL OF PORTAL HYPERTENSION

1. Materials and methods

SECTION C. BASIC METHODS

1. Anaesthesia

2. Measurement of mean arterial pressure and portal venous pressure

3. Measurement of portasystemic shunting

DISCUSSION

INTRODUCTION

This chapter is divided into three sections. The first section describes both the animals used during the study period and the environment in which they were housed. A basic outline of the relevant rat anatomy is given and emphasis is placed on the anatomy of the splanchnic circulation.

The second section describes an experimental rat model of prehepatic portal hypertension which is produced by creating a calibrated resistance to portal venous flow (Myking and Halvorsen, 1973). Various animals and techniques have been used over the years in an attempt to reproduce in an experimental model the clinical syndrome of portal hypertension (Childs et al, 1950; Rozga et al, 1985). The rat and the technique of partial portal vein ligation have emerged as the most favourable in establishing a prehepatic model of portal hypertension. Although rat models of hepatic cirrhosis are available (McLean et al, 1969; Proctor and Chatamra, 1982) and would more closely reproduce the clinical syndrome in its most commonly encountered form, agents such as galactose which are normally extracted by the liver with high efficiency may pass through sinusoids in the cirrhotic liver with relatively little extraction. This "functional shunting" is difficult to quantify with an independent

measurement technique. In prehepatic portal hypertension, liver function is normal and shunting occurs through relatively large calibre extrahepatic collateral vessels. This "anatomical shunting" can be independently measured, and a prehepatic model therefore provides a better basis for validating the oral galactose technique as a measure of shunting.

The choice of model was based upon work carried out by Geraghty et al (1989) in which the relationship between portal venous pressure and shunting of mesenteric venous blood was examined in the rat prehepatic model of portal hypertension as described by Myking and Halvorsen (1973). Ideally for this study the choice of model should be one that demonstates a variable degree of shunting. Geraghty and colleagues have demonstrated that the Myking and Halvorsen (1973) prehepatic model of portal hypertension is associated with highly variable degrees of shunting from the mesenteric vascular bed, with no clear relationship between individual values of portal venous pressure and shunting during the three to seven day period following portal vein ligation. Such observations have led to experiments in this study being conducted 6 to 8 days following portal vein ligation, a point in time where gross haemodynamic changes in splanchnic blood flow are minimised and portasystemic shunting has largely become evolved.

The third section in this chapter describes the anaesthetic agents used throughout the experimental period, and the technique used to measure mean arterial pressure and portal venous pressure, both of which are recorded in most haemodynamic studies, is also outined in this section. Further, the use of gamma-labelled microspheres in quantitating the magnitude of portasystemic shunting as described by Chojkier and Groszmann (1981) is also detailed in this section.

SECTION A. BASIC MODEL

ANIMALS AND HOUSING

In all experiments male Sprague-Dawley rats (Banton and Kingman limited, Hull) were used. All were housed in stainless steel cages, with a maximum of four rats per cage. The animals were fed on a standard rat diet (RNM, Type 1) with water ad libitum, and exposed to a 12 hour light/dark cycle, a relative humidity of 50 ± 5 percent and a temperature range of 21 ± 1 degrees C as implemented in the animal facility. Twenty-four hour fasting prior to experimentation was carried out by housing an individual rat in a wire bottom stainless steel cage during which time water was freely available.

RELEVANT RAT ANATOMY

Liver

The gross anatomy of the rat liver is comparable to that in man. The rat liver is situated below the right hemidiaphragm in the peritoneal cavity, and consists of the left lateral, right lateral, median and caudate lobes. Its blood supply is via the portal vein and the hepatic artery, and it is drained by two or three hepatic veins into the inferior vena cava. As

in man the liver is divided into hepatic lobules which contain central venules, hepatic sinusoids, hepatocytes and portal vessels.

Portal Circulation

The portal vein drains the intestinal tract from the stomach to the upper rectum and is formed by the union of the superior mesenteric, splenic and pyloric veins. The superior mesenteric vein is formed by the union of approximately fifteen intestinal branches, the ileo-colic, right colic and middle colic veins (Figure 13).

ANATOMY OF PORTAL VENOUS SYSTEM OF THE RAT

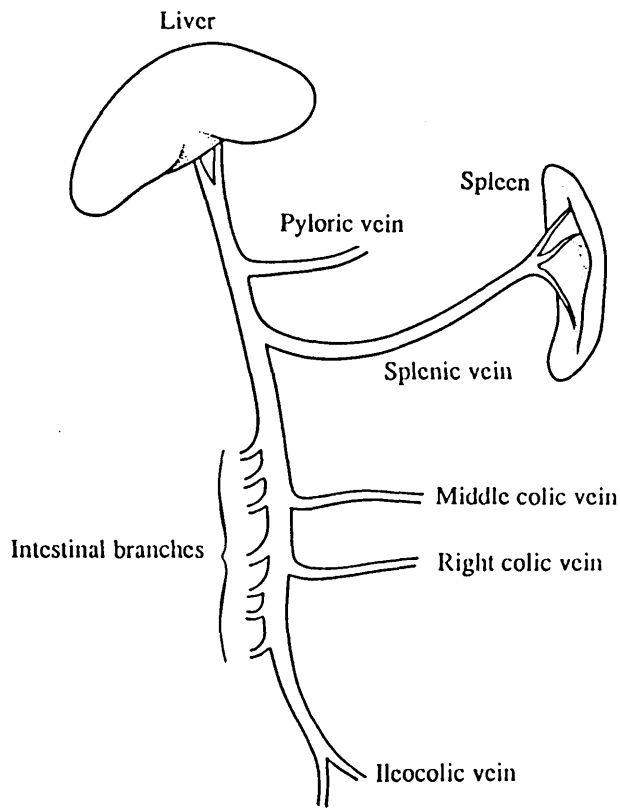


Figure 13

The ileocolic branch of the portal vein was cannulated for measurement of portal venous pressure, infusions of galactose into the portal vein, and quantitation of the magnitude of portasystemic shunting by injecting radioactive microspheres into the portal venous system via the ileocolic cannula.

SECTION B. A PREHEPATIC MODEL OF PORTAL HYPERTENSION

With reference to the introduction, the following text describes the experimental rat model of prehepatic portal hypertension.

MATERIALS AND METHODS

Animals

In all experiments male Sprague-Dawley rats weighing between 250 and 390 grams were used. A maximum of four animals were housed in each cage, which contained on average an equal number of sham operated and portal vein ligated animals.

Technique of portal vein ligation

A rat model of prehepatic portal hypertension was established by creating a calibrated resistance to portal venous flow using the portal vein ligation technique (Myking and Halvorsen, 1973). Under light ether anaesthesia animals were subjected to laparotomy. The liver and intestines were then expelled from the abdominal cavity and retracted to enable clear visualisation of the portal vein. The portal vein was mobilised over a length of 1cm just distal to its bifurcation into the right and left hepatic branches.

In all cases it was important to ensure that the hepatic artery which lies adjacent, was separated from the portal vein. A 21 gauge needle (OD = 0.86mm) with a blunt tip was placed alongside the mobilised portion of the portal vein and a 3/0 silk tie placed around both the portal vein and the needle and secured. A calibrated resistance to portal venous flow was created on removal of the needle (Figure 14). The liver and intestines were then returned to the abdominal cavity and the incision closed in two layers. In sham operated controls the portal vein was mobilised but not stenosed.

TECHNIQUE OF PORTAL VEIN LIGATION

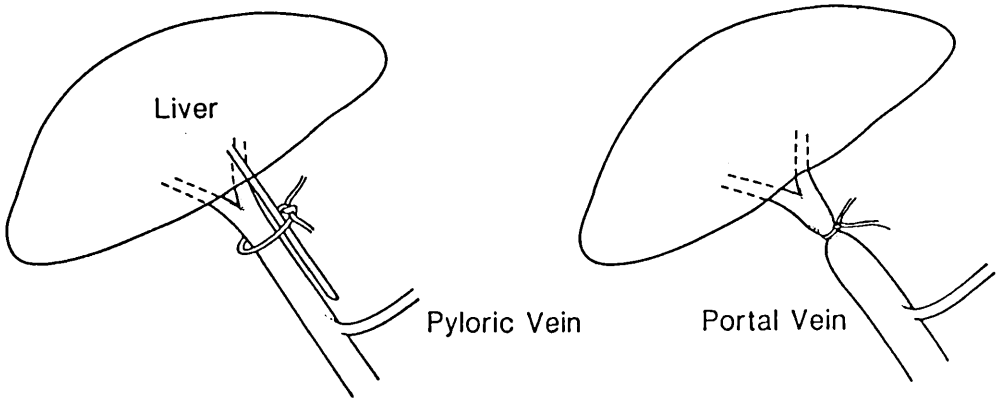


Figure 14

A 21 gauge needle was placed alongside the mobilised portal vein and a 3/0 silk tie placed around both vein and needle. Removal of the needle resulted in a calibrated resistance to portal venous flow.

SECTION C. BASIC METHODS

The following text describes the anaesthetic agents used throughout experimental procedures, and the technique used to measure mean arterial pressure and portal venous pressure. Further, this section describes microspheres and the injection of radioactive microspheres in the quantitation of portasystemic shunting (Chojkier and Groszmann, 1981).

ANAESTHESIA

A. ETHER

Animals while undergoing the recovery procedure of partial portal vein ligation, were subjected to ether anaesthesia maintained throughout surgery by means of a 50ml flask containing etherised cotton wool.

B. NITROUS OXIDE : OXYGEN : HALOTHANE

All haemodynamics studies were conducted using a nitrous oxide, oxygen, halothane mixture. Induction was carried out by delivering to a perspex box 4% halothane in a 2:1 nitrous oxide : oxygen mixture at a 0.6 litres per minute flow rate. Following induction a tracheostomy was performed (5 French Gauge Portex

tubing) and the animal ventilated using a Harvard rat ventilator (Model 680) which maintained a respiratory rate of 35-45 strokes per minute, and a tidal volume of 3.0-4.0ml. Ventilation was maintained with 0.5%-1.0% halothane in the same nitrous oxide : oxygen mixture. The adequacy of the depth of anaesthesia was guaged by the absence of response to painful stimuli, and occassionally it was necessary to increase halothane concentration by up to 1% to achieve this. Blood gas analysis was performed with a regularly-calibrated automatic analyser (ABL 2, Radiometer) on a volume of 0.3ml arterial blood drawn from the right femoral artery cannula and experiments were only performed when a p02 over 90mmHg, pC02 between 35-45mmHg and a pH over 7.30 could be maintained throughout the experimental period. Occasionally minor adjustments in respiratory rate were necessary to maintain pC02 within the above range. A rectal thermometer monitered core temperature, which was maintained at 37 $\pm$ 0.5 degrees C by means of heating lamp.

C. PENTOBARBITOL

Intraperitoneal pentobarbitol was used in some initial oral galactose experiments. 60mg/kg body weight of pentobarbitol was injected into the peritoneal cavity with a 25 gauge needle. Adequate anaethesia was

provided with this dose for approximately 90 minutes and if neccesary it was supplemented with 0.2mg/kg of pentobarbitol.

MEASUREMENT OF MEAN ARTERIAL PRESSURE

On commencing artificial ventilation, the animal was placed supine on the operating table and a transverse groin incision made, followed by isolation of the femoral artery. Cannulation was performed with a polyethelene catheter (outside diameter = 0.96 mm, inside diameter= 0.51mm) which was then connected to a (Statham) strain gauge transducer and a Gould series 800 recorder which recorded arterial pressure. The recorder noted systolic, diastolic and mean arterial pressure readings, and only animals with a mean arterial pressure of at least 90mmHg were included in all haemodynamic studies. Each animal was heparinised (130 units/kg body weight) on placement of the femoral artery cannula as clotting of the cannulae was found to impede pressure tracings.

Some experiments necessitated the cannulation of both femoral arteries, a femoral vein and the portal vein. In all cases the same type of cannula was used.

MEASUREMENT OF PORTAL VENOUS PRESSURE

Portal venous pressure was measured directly in all haemodynamic studies by cannulation of the portal vein. A lower mid-line abdominal incision was made following anaesthesia, and the caecum and the distal

small bowel delivered to the surface of the abdomen. The intestines were wrapped in a saline soaked swab and the ileo-colic branch of the portal vein isolated and cannulated (Polyethelene cannula, OD = 0.96mm). The cannula was positioned approximately 3cm into the superior mesenteric vein and secured in place with a 3-0 silk tie. The abdomen was closed and the cannula was connected to a strain gauge transducer to record portal venous pressure as previously described for arterial pressure.

CALIBRATION OF TRANSDUCERS

Calibration of both arterial and venous transducers were performed before each experiment. The zero balance and calibration was re-checked again before formal pressure readings were recorded. A mercury manometer using a 100mmHg column was used to calibrate the strain guage transducer measuring mean arterial pressure.

Calibration of portal venous pressure was performed by use of a specially constructed manometer with a column of water equivalent to 25mmHg, this allowed precise calibration of the portal venous strain guage. Measurement by use of the mercury column proved to be inaccurate in the calibration of the portal venous

pressure due to the different range settings. Portal venous pressures were recorded approximately 20 minutes after abdominal closure and when a respiratory pattern was present on the portal venous tracing. A final portal venous pressure measurement, therefore required accurate calibration of the strain guage transducer, correct zero balance readings before and after pressure measurements, normal blood gases and a respiratory pattern on the portal venous tracing.

THE USE OF GAMMA-LABELLED MICROSPHERES TO QUANTITATE THE MAGNITUDE OF PORTASYSTEMIC SHUNTING

Before describing the measurement of radioactive microspheres in quantitating portasystemic shunting a resume of the nature of microspheres themselves and potential problems in their use in quantitating portasystemic shunting is given.

A. Physical Properties of Radioactive Microspheres

Microspheres are resin-coated spherical beads which are manufactured in various sizes and can be labelled with a wide range of radioisotopes. The radioisotopes contain unstable nuclei and emit differing forms of radiation, including gamma rays, during the process of radioactive decay. The magnitude of portasystemic shunting was quantitated by use of microspheres labelled with Cobalt-57.

The microspheres used in the study had a diameter of 15 $\pm$ 1 microns (mean $\pm$ SD) whereas rat capillaries have a mean diameter of approximately 8 microns (Gannon and O'Brien, 1982). Therefore the microspheres will be trapped in rat capillaries following intraportal injection. Further, if the microsperes mix uniformly with blood, then their distribution to target organs following injection will give an index of the

distribution of blood flow from the injection site to that organ. All microspheres injected into the portal vein in normal rats without portasystemic shunting will be trapped in the hepatic sinusoids. If portasystemic shunting is present, however, microspheres will bypass the liver in proportion to the degree of diversion of portal venous blood into the systemic circulation and become trapped in lung capillaries. An index of the magnitude of portasystemic shunting is thus given by the ratio of microspheres in lungs to that in liver and lungs.

B. Sources of error when using radioactive microspheres to quantitate the magnitude of portasystemic shunting

A number of physical properties of a radioactive microsphere exist which may lead to inaccuracies in quantitating the magnitude of portasystemic shunting.

i) Aggregation of microspheres

Microspheres readily adhere and clump together despite the presence of a polymeric coat. This physical property can therefore give rise to large sources of error when measuring the degree of portasystemic

shunting. Clumping of microspheres can be easily checked by placing a drop of microsphere suspension on a slide and inspecting it for clumping under light microscopy.

Microspheres are prepared by suspension in a high molecular weight solution (10% dextran) to which 0.01% of the surfactant "Tween"-80 (BDH) is added to minimise clumping. However despite this, microsphere suspensions aggregate when left standing for prolonged periods of time. Therefore in order to minimise clumping of microspheres both the container, and subsequently the syringe containing the microspheres are vortexed immediately prior to injection.

ii) Leaching

The separation of radio-isotope from the microsphere is known as leaching. If leaching occurs the radio-isotope will circulate freely in the systemic circulation while microspheres will continue to be trapped by target organs such as the liver. Elevated levels of radioactivity in the lungs and kidneys following intraportal injection of radioactive microspheres in control animals is indicative of leaching. Regular checks on the microsphere suspension were performed by centrifugation of the microsphere suspension and measuring radioactivity in the

supernatant fluid relative to that in the sedimented microspheres. Microspheres were resuspended in fresh dextran if greater than 1% of total radioactivity had leached. As a quality control procedure in each experiment the kidneys were removed and counted to ensure that radioactivity was not greater than that of background levels.

MEASUREMENT OF RADIOACTIVE MICROSPHERES IN QUANTITATING PORTASYSTEMIC SHUNTING

On completion of each experiment, when formal recordings for mean arterial pressure, arterial blood gases, portal venous pressure and rectal temperature had been performed as previously described, a vial containing radioactive microspheres (New England, Nuclear) was vortexed for 30 seconds and a 0.3ml suspension containing in excess 500,000 gamma-labelled microspheres was drawn into a 1ml syringe. The syringe, needle and cap were vortexed for a further 20 seconds immediately prior to injection into the portal vein via the ileo-colic cannula. The cannula was then flushed with 0.5ml of normal saline. Approximately three minutes following intraportal injection of radioactive microspheres, the animal was sacrified by opening the chest, and liver, lungs and kidneys were removed. The liver, lungs and kidneys were each sliced individually into approximately 1 cm long segments and then placed in counting vials for radioactivity maesurements. This was performed by use of a Gamma Scintillation counter (Packard Instruments, Auto-gamma 5000) which contained a sodium iodide crystal. Measurements were made using an open energy window (15-2000KeV). The magnitude of portasystemic shunting was calculated by use of the following formula;

$$\text{Portasystemic shunting} = \frac{\text{Lung Radioactivity (dpm)}}{\text{Liver + Lung Radioactivity (dpm)}}$$

.....5.1

(Chojkier and Groszmann, 1981)

Each animal was checked to ensure absence of significant leaching or lung shunting by assuring that kidney counts were no higher than background levels.

DISCUSSION

ANIMAL MODEL

Many attempts have been made to reproduce in experimental animals, the clinical syndrome of portal hypertension as seen in man. Results have been disappointing in both the dog (Seiro et al, 1963) and monkey (Childs et al, 1950) as collateral vessels sufficient to decompress the portal vein readily develop. The rat, however, has proved to be a better model of portal hypertension, in so much that portal venous pressure has been demonstrated to remain elevated for at least eight weeks following portal venous stenosis despite the presence of portasystemic collateral vessels (Halvorsen and Myking, 1979).

A variety of techniques have been used in the rat to produce prehepatic portal hypertension. Such techniques include ameroid constriction of the portal vein (Saku et al, 1976), placement of a silastic button around the portal vein (Rozga et al, 1985) and simple partial ligation of the portal vein (Myking and Halvorsen, 1973). Although the technique of portal vein ligation is in practise relatively easy, a fine balance does exist between the degree of stenosis of the portal vein and subsequent mortality. In 1973 Myking and Halvorsen examined the relationship between animal survival and the degree of stenosis and

demonstrated that 1.0mm stenosis of the portal vein resulted in almost 100% mortality due to mesenteric vein thrombosis. In contrast at 1.2mm stenosis reduced mortality to 25%. Since the degree of stenosis and so mortality will vary with the diameter of the portal vein and thereby with the weight of the animal, it was necessary in this study to use animals within a narrow weight range (250 to 390 grams). This range negated the need for different gauge needles with varying animal weights and avoided any influence that animal age may have on the development and course of portasystemic shunting.

The need for a prehepatic model of portal hypertension which is associated with a wide range of portasystemic shunting is fundamental to this study. Only with a wide range of portasystemic shunting can the ability of galactose to accurately measure all magnitudes of portasystemic shunting be addressed. The prehepatic model of portal hypertension has been shown to be associated with a wide spectrum of portasystemic shunting, with values ranging from zero to 85% as early as one day after portal vein ligation (Geraghty et al, 1989). This variation continued and at 7 days after portal vein ligation, a time period which was comparable with that of this study, mean portasystemic shunting was 60.4 $\pm$ 8.8 percent (Geraghty et al, 1989). The technique used to quantify the magnitude of

portasystemic shunting in experimental models of portal hypertension was first described by Chojkier and Groszmann (1981). The model so described by Chojkier and Groszmann (1981) involved intrasplenic injection of radioactive microspheres and was associated with a narrow range, 89 percent to 99 percent of portasystemic shunting ten days following portal vein ligation. Indeed by means of this technique, blood from the splenic vascular bed has consistently been found to be almost totally shunted in rats with established prehepatic portal hypertension (Benoit et al, 1985; Groszmann et al, 1982). In contrast, the model described by Geraghty et al (1989) involved injection of radioactive microspheres into the portal vein. From this the mesenteric contribution to portal venous inflow was found to be subject to lower and more variable degrees of shunting. The model so described by Geraghty et al (1989) would thus appear to be more suitable for the needs of this study. However, it must be stressed that shunting of microspheres injected at any given point will not mirror exactly the shunting of blood from all tributaries of the portal vein, but administration via the superior mesenteric vein ensures that they are mixed with the dominant contribution of portal venous inflow.

By virtue of this type of rat model of portal hypertension, which was associated with a wide range of

portasystemic shunting, Geraghty et al (1989) were able to carry out a comparative study between the magnitude of portasystemic shunting and corresponding level of portal venous pressure at various time intervals following portal vein ligation. Since it is generally accepted that shunts develop as a response to increased portal venous pressure, it might be expected that there would be a direct relationship between the level of pressure and the degree of shunting to which it gives rise. However, as the effect of shunting is to decompress the portal vein, equally plausable is the idea of an inverse relationship in which high degrees of shunting are associated with low pressure. An inverse relationship between pressure and shunting could occur if factors other than pressure, such as anatomical variation, control the development of shunts. In the study carried out by Geraghty et al (1989) an initial increase in shunting and a progressive fall in portal venous pressure with time was demonstrated, however no clear relationship between individual values of pressure and shunting during a 3 to 7 day period could be established. Further, at later time intervals a direct relationship between portal venous pressure and portasystemic shunting was established wherein increasing degrees of shunting were associated with increasing levels of portal venous pressure. The relationship between portal venous

pressure and portasystemic shunting is in itself poorly understood. The variation in the magnitude of portasystemic shunting and thus the differing relationships between pressure and shunting as seen in the study by Geraghty et al (1989) could be attributed to the ability to open portasystemic shunts in response to an initial elevated portal venous pressure, the anatomical variation in the site of these shunts and the inter-operator variability while performing the technique of partial portal vein ligation. However, the direct relationship between portal venous pressure and portasystemic shunting is consistent with the idea that pressure is the driving force for the evolution of shunts. Alternative interpretations of this direct relationship between portal venous pressure and portasystemic shunting have also been proposed (Benoit et al, 1985; Vorobioff et al, 1983; Blanchet and Lebrec, 1982).

The idea that anatomical variations in the site of portasystemic shunts may be a factor in controlling the development of collaterals, would appear to be doubtful. Investigators have repeatedly found that on stenosis of the portal vein the development of portasystemic collaterals show little variability in the their site and quantity (Bengmark et al, 1976; Halvorsen and Myking, 1974).

In patients with portal hypertension relatively little

is known about the relationship between magnitude of portasystemic shunting and level of portal venous primarily because of the difficulty of measuring these parameters non-invasively. Most of the few studies in which shunting has been quantified in humans have failed to demonstrate any definate correlation with portal pressure (Caesar et al, 1962; Groszmann et al, 1972).

ANAESTHESIA

The use of anaesthetic agents in any experimental animal work must achieve two objectives. The first, and most obvious is in providing an adequate level of anaesthesia and the second is in maintaining normal cardio-respiratory function throughout the anaesthetic period. Many anaesthetic agents have been described which provide small animals with an adequate level of anaesthesia, however in parallel these agents have been associated with inducing abnormal cardiac or respiratory function. For example, the irritant properties of ether, which can result in respiratory difficulties sufficiently pronounced to induce respiratory arrest has limited its experimental use to short spanned recovery procedures in which measurements of systemic haemodynamics were not necessary. Fluctuating levels of anaesthesia were also experienced

in pilot studies using intraperitoneal pentobarbitol.

To a large extent the experimental animal work carried out in this thesis was haemodynamic in nature, therefore necessitating a choice of anaesthetic which would produce a minimun derangement in cardio-respiratory function. This was particularly relevent in the measurement of portal venous pressure and the measurement of the magnitude of portaystemic shunting where alterations in splanchnic inflow would affect both parameters. Cooperman (1972) demonstrated that splanchnic inflow was affected by the partial pressure of carbon dioxide, with hypercapnia producing splanchnic vasoconstriction and diminished splanchnic blood flow while hypocapnia produced the opposite effect. Particular emphasis was therefore placed on early pilot work in which tidal volume and respiratory rates associated with normal arterial blood gases were determined.

Depression of the vasomotor centre frequently occurs on usage of most anaesthetic agents, therefore the effect of halothane on systemic and regional haemodynamics is of particular interest. Examination of the effects of a 1 and 2% halothane mixture with oxygen on left ventricular function in dogs and primates was carried out by Vatner and Smith (1974). A concentration dependent depression of myocardial contractility with a fall in mean arterial pressure from 100 to 86mm Hg was

observed in dogs anaesthetised with 1% halothane, the effect being further accentuated in dogs receiving 2% halothane. In parallel studies carried out by Hughes et al (1980) halothane reduced to 0.5% did not result in a significant reduction in cardiac output, however on increasing inspired halothane to 1.5% a significant depression in cardiac output was observed. A dose dependent decrease in portal venous blood flow was also observed, whereas no significant fall in total peripheral resistance with 0.5% halothane occured. Futher, increases in halothane concentration from 0.5 to 2% did not significantly alter portal venous pressure. It would thus appear from the collated evidence that high levels of inspired halothane depress myocardial function, whereas in contrast, a concentration of 0.5% halothane produced minor effects on systemic and splanchnic haemodynamics. Subsequently the level of inspired halothane used in all animal experiments rarely exceeded 0.5%.

Splanchnic haemodynamics are also affected by animal core temperature, and heat loss invariably occurred during cannulation of the portal vein. A minimum period of 30 minutes therefore elapsed between abdominal closure and measurement of portal venous pressure. Further, experiments were initiated only when a core temperature of 37 $\pm$ 0.5 degrees C was recorded. Therefore by minimising factors known to

affect systemic and splanchnic haemodynamics a stable experimental animal preparation was achieved.

CHAPTER SIX GASTRIC GALACTOSE ABSORPTION AND INTRAPORTAL INFUSION OF GALACTOSE IN AN EXPERIMENTAL MODEL OF PREHEPATIC PORTAL HYPERTENSION

SECTION A. OROGASTRIC ADMINISTRATION OF GALACTOSE

1. Introduction
2. Materials and methods
3. Results
4. Discussion

SECTION B. CARBON-14 LABELLED GALACTOSE

1. Introduction
2. Materials and methods
3. Results
4. Discussion

SECTION C SINGLE INFUSION OF GALACTOSE INTO THE PORTAL VEIN

1. Introduction
2. Materials and methods
3. Results
4. Discussion

CONCLUSION

SECTION A. OROGASTRIC ADMINISTRATION OF GALACTOSE

INTRODUCTION

The aim of the following experiments were to establish an oral dose of galactose, that following absorption from the small intestine was comlpletely cleared from portal blood on its first pass through the liver in control animals.

In order to establish a liver threshold dose for first pass clearance of galactose, descending oral doses of galactose were administered to sham operated rats and systemic blood galactose concentrations measured by galactose dehydrogenase enzyme assay, as described in Chapter 3. Once established, this liver threshold dose was orally administered to portal vein ligated animals, and blood galactose concentrations measured as already described.

The areas under the galactose appearance curve, and so systemic availability from these blood galactose time graphs were calculated in both control and portal hypertensive rats. The technique used and the results are described in this section.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 310 ± 79.1 grams (mean ± SD) were used. Sham operation and portal vein ligation was performed as described in Chapter 5. All animals were fasted for 24 hours but allowed free access to water. Anaesthesia was induced and the animal ventilated as previously described in Chapter 5. Both femoral arteries were cannulated and arterial blood samples were taken for blood gas analysis. Arterial pressure was monitored continuously via the left femoral artery cannula and core temperature was maintained at 37 ± 0.5 degrees C by means of a heat lamp. Before gavage an arterial blood sample was collected to determine basal galactose concentration. All blood samples were analysed as described in Chapter 3.

Galactose was administered via a 6 French guage portex infants feeding tube which was advanced from the mouth into the stomach. A water gavage of 2mls was administered approximately twenty minutes before the galactose in order to empty any residual stomach contents. D-galactose dissolved in distilled water to a concentration of 40 mg/ml was used to prepare doses in the range 25 to 130 mg/kg body weight, which were flushed into the stomach of the animal. The gavage tube was removed and timed arterial blood samples collected

every 10 minutes, from the right femoral artery cannula (Figure 15). An increase in blood pressure occurred on gavage which almost immediately returned to normal on withdrawal of the tube. All experiments were terminated if blood pressure fell below 80mmHg. However, no experiments were discarded as it was found on enzyme analysis, that in all cases, systemic galactose concentrations had reached a maximum and were returning towards basal levels as arterial pressure fell below 80 mmHg. If arterial pressure remained above 80 mmHg, blood samples were collected until systemic galactose concentrations had returned to basal levels. Early experiments showed this to occur between two and two and a half hours after the initial gavage.

At the end of the experiment the animal was killed by opening of the chest. In all cases the stomach of the animal was removed and examined for the presence of food.

The aim and expectation of these experiments was to demonstrate a significant difference in systemic availability of oral galactose between portal hypertensive and control rats, as had been found readily in patients (Chapter 4). The extensive blood sampling procedure placed a heavy load on the circulation, and it was found in pilot experiments that the additional stress produced by the procedure of portal vein cannulation made it difficult to maintain

an adequate level of arterial pressure. As previously discussed in chapter 5 the technique of portal vein ligation applied in this study produced portasystemic shunting in excess of 50% with high reliability (Geraghty et al, 1989), therefore portal vein cannulation and measurement of portasystemic shunting was dispensed with in all of these experiments.

GALACTOSE GAVAGE AND SAMPLING TECHNIQUE

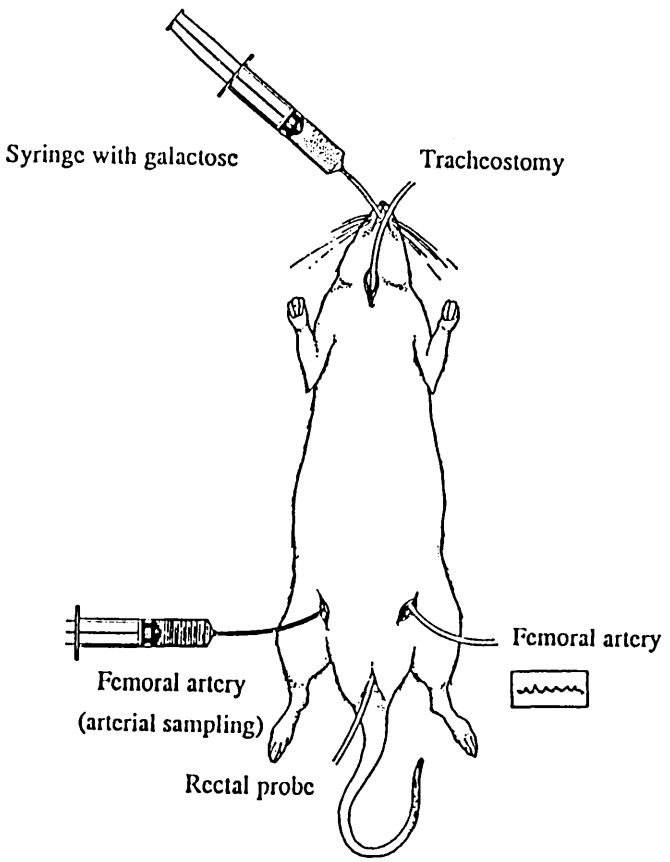


Figure 15

Oral administration of galactose in rats was performed by flushing concentrations of galactose into a 6 French gauge portex infants feeding tube which had been advanced from the mouth into the stomach. Timed arterial samples were collected from the right femoral artery and analysed as described in Chapter 3.

Design of study

In an attempt to discover the liver threshold dose of galactose, descending doses of galactose, 130 mg/kg to 25 mg/kg were orogastrically administered to sham operated rats. Six rats received 130 mg/kg of galactose. The dose was then reduced to 100 mg/kg, 50 mg/kg and finally 25 mg/kg, with two, two and nine sham operated rats receiving the dose respectively.
After provisionally accepting a liver threshold dose for first pass clearance of galactose in sham operated rats, seven portal vein ligated rats were orogastrically administered doses of 25 mg/kg.

AUC calculation and Statistical analysis

In all experiments, areas under the galactose appearance curve (AUC) from concentration time graphs were determined by a specially written computer program, using linear interpolation between points, and the statistical significance of observed differences between sham operated and portal vein ligated animals was tested by the Student's t-test. In calculating AUC a standardised total time of sampling of 70 minutes was imposed in all experiments. The total AUC, however, was also calculated in experiments which exceeded 70 minutes. The area under the curve divided by its

corresponding dose (AUC/dose) at both 70 minutes and in total was also calculated.

The expectation of increasing AUC/dose with an increasing dose is shown by the following equations:

With reference to Chapter 2 and by rearranging equation 2.13,

$$AUC/Q = SA/K = S + (1-S)(1-E)/K \quad \ldots\ldots\ldots\ldots 6.1$$

Where,

Q = Oral dose of galactose
SA = Systemic availability of galactose
K = Systemic galactose clearance
E = Hepatic extraction efficiency of galactose

If absorption of galactose from the gut is not complete then,

$$AUC/Q = A.\{S + (1-S)(1-E)\}/K \quad \ldots\ldots\ldots\ldots\ldots 6.2$$

in which the term A represents that fraction of galactose which has been absorbed from the gut.

From equations 2.10 and 2.11, (Chapter 2),

$$K = E.F \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 6.3$$

Where E represents hepatic extraction efficiency, and F represents hepatic blood flow, and extrahepatic clearance of galactose is assumed to be negligible.

Hence,

$$AUC/Q = A.\{S + (1-S)(1-E)\}/E.F \quad \ldots\ldots\ldots\ldots 6.4$$

In sham operated rats, shunting is zero (S=0), and

$$AUC/Q = A.(1-E)/E.F \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 6.5$$

Equation 6.5 shows us that with increasing dose, AUC should not increase merely in proportion. The ratio AUC/Q should also increase if, as a result of the higher blood galactose concentration attained, hepatic extraction efficiency, E, decreases.

RESULTS

Total area under the galactose appearance curve (AUC total), area under the galactose appearance curve at 70 minutes (AUC t=70min), blood galactose appearance peaks and corresponding appearance peak times, AUC/dose total, and AUC/dose t=70min in individual sham operated rats are tabulated against oral galactose doses in Table 11. A clearly defined blood galactose appearance peak was seen in all rats who received at least 50 mg/kg galactose orally, and in 6 out of 9 rats who received an oral dose of 25 mg/kg (Table 11). In all but five cases a single blood galactose appearance peak was seen. In all but 2 rats a first blood galactose appearance peak reached a maximum level between 10 and 40 minutes, and of these, five showed a second peak to occur between 50 and 102 minutes following administration of the oral dose. In the case of a single blood galactose appearance peak, galactose levels in most animals returned towards basal level 70 minutes after administration of the oral dose, but if a second peak occurred, basal levels were not reached until 110 minutes. In two rats the maximum level of the second blood galactose peak exceeded that of the first peak, and in a third rat both peaks reached the same maximum level (Table 11).

In three rats who received 25 mg/kg galactose, there

was no well-defined appearance peak, and the maximum concentration recorded in blood did not exceed 5 mg/kg (Table 11).

In three out of six rats who received 130 mg/kg, 1 out of 2 who received 50 mg/kg and 2 out of 9 who received 25 mg/kg, some residual food was found in the stomach on completion of the experiment. The expectation of delayed absorption and reduced areas under the curve in the presence of food was not consistently found, indeed absorption of galactose from the stomach and areas under the galactose appearance curve were variable regardless of whether the stomach contained food or not (Table 11).

Descending oral doses of galactose were accompanied with decreasing systemic blood concentrations in sham operated rats. Areas under the galactose appearance curve 70 minutes following gavage (AUC t=70min) in the range 140 to 6375 mg.min/l, 1510 and 3140 mg.min/l, 196 and 1345 mg.min/l, and -180 to 655 mg.min/l at oral galactose doses of 130, 100, 50, and 25 mg/kg respectively are shown in Figure 16. The relationship between area under the galactose appearance curve (AUC) and oral galactose dose (GD) was described by the linear regression equation AUC = 28.12GD - 514.35. A correlation coefficient of 0.698 was found. The mean $\pm$ SD values for AUC t=70min were 228 $\pm$ 263 mg.min/l, 771 $\pm$ 813 mg.min/l, 2325 $\pm$ 1153 mg.min/l and 3159 $\pm$ 2538

mg.min/l at oral doses of 25, 50, 100, and 130 mg/kg respectively (Table 12).

In Table 12 the mean ± SD for AUC total, AUC t=70min, AUC/dose total, AUC/dose t=70min are tabulated against oral galactose concentrations of 25, 50, 100, 130 mg/kg.

At increasing oral doses, AUC/dose showed an increasing trend. Individual values of AUC/dose t=70min ranged from 1.1 to 49.0, 15.1 and 31.4, 3.6 and 24.5, and -7.2 to 26.2 kg.min/l at oral galactose doses of 130, 100, 50, and 25 mg/kg respectively (Table 11). The mean ± SD for AUC/dose t=70min was 24.3 ± 19.5, 23.3 ± 11.5, 14.1 ± 14.8, and 9.2 ± 10.5 kg.min/l at oral doses of 130, 100, 50, and 25 mg/kg respectively (Table 12). However, the difference between values was not statistically significant. A similar pattern was found for total AUC/dose.

The pattern of systemic galactose appearance, and the corresponding AUC total and AUC t=70min from different rats at the same oral concentration of galactose were very variable (Table 11). Figure 17 shows the variability between systemic galactose appearance curves, and so area under the curve in two sham operated rats after orogastric administration of galactose at a dose of 130 mg/kg.

At oral galactose doses of 25 mg/kg, systemic galactose concentrations approximated basal levels of galactose

and thus the liver threshold dose for first pass clearance of galactose in sham operated rats was provisionally taken to be 25 mg/kg.

A well-defined blood galactose appearance peak was seen in only three out of seven portal vein ligated rats who received an oral dose of galactose of 25 mg/kg (Table 13). In only one rat was a second blood galactose appearance peak seen, which exceed the first peak by 3 mg/l. Blood galactose reached a maximum level 10 to 50 minutes after administration of the oral dose, declining towards basal levels after 60 minutes. The maximum concentration in blood did not exceed 70 mg/l above baseline, and the minimum was 2 mg/l. In only one rat was food found in the stomach on completion of the experiment.

Gavage with 25 mg/kg of galactose in portal vein ligated rats produced an area under the curve at t=70min no greater than 890 mg.min/l (Table 13), with a mean $\pm$ SD of 257 $\pm$ 313 mg.min/l (Table 12). No significance difference was found in AUC t=70min values between portal vein ligated and sham operated rats at an oral galactose dose of 25 mg/kg (p=0.844). Similarly, no significant difference in total AUC values between portal vein ligated and sham operated rats at an oral galactose dose of 25 mg/kg (p=0.927) was found.

AUC/dose at 70 minutes ranged from -1.8 to 35.6

kg.min/l at an oral dose of 25 mg/kg (Table 13), with a mean ± SD of 10.3 ± 12.5 kg.min/l (Table 12). AUC/dose total ranged from 7.6 to 35.6 kg.min/l at an oral dose of 25 mg/kg (Table 13), with a mean ± SD of 16.9 ± 9.1 kg.min/l (Table 12). No significant difference was found in AUC/dose total, and AUC/dose t=70min between portal vein ligated rats and sham operated rats, p=0.936 and p=0.850 respectively.

TABLE 11

Dose (mg/kg)	Duration of sampling (mins)	AUC total (mg.min/l)	AUC t=70 (mg.min/l)	CGmax 1 (mg/l)	CGmax 2 (mg/l)	Tpk 1 (mins)	Tpk 2 (mins)	AUC/dose total (kg.min/l)	AUC/dose t=70 (kg.min/l)
130	190	2215	1695	45	28	10	50	17.0	13.0
130(i)	152	535	140	7	7	22	102	4.1	1.1
130(i)	70	2003	2003	64	-	30	-	15.4	5.4
130	70	6375	6375	190	-	30	-	49.0	49.0
130	140	8305	2590	118	-	90	-	63.9	19.9
130(i)	70	6152	6152	131	-	30	-	47.3	47.3
100	150	2400	1510	47	49	30	70	24.0	15.1
100	150	4405	3140	90	-	60	-	44.5	31.4
50	90	334	196	5	-	30	-	6.1	3.6
50(i)	150	1930	1345	35	-	40	-	35.1	24.5
25(i)	150	310	50	8	5	10	90	12.4	2.0
25(i)	100	675	465	10	13	10	60	27.0	18.6
25	70	475	475	13	-	20	-	19.0	19.0
25	150	500	120	5	-	(a)	-	20.0	4.8
25	110	380	200	8	-	20	-	15.2	8.0
25	150	1180	655	21	-	10	-	47.2	26.2
25	110	340	245	10	-	10	-	13.6	10.2
25	110	165	-180	3	-	(a)	-	6.6	-7.2
25	110	-115	25	0	-	(a)	-	-4.6	1.0

Individual AUC_{total} and other expressions have been calculated in sham operated rats at different oral galactose doses. The above calculated expressions are defined on the following page.

The definitions of the calculated expressions in Table 11 are shown below;

AUC_{total} = Total area under the galactose appearance curve

$AUC_{t=70}$ = Area under the galactose appearance curve from 0 to 70 minutes

CGmax = Maximum blood galactose concentration after oral dose

Tpk = Time of blood galactose appearance peak

$AUC/dose_{total}$ = Total systemic availability of galactose

$AUC/dose_{t=70}$ = Total systemic availability of galactose from 0 to 70 minutes

(i) = Food present in stomach

(a) = No definable peak

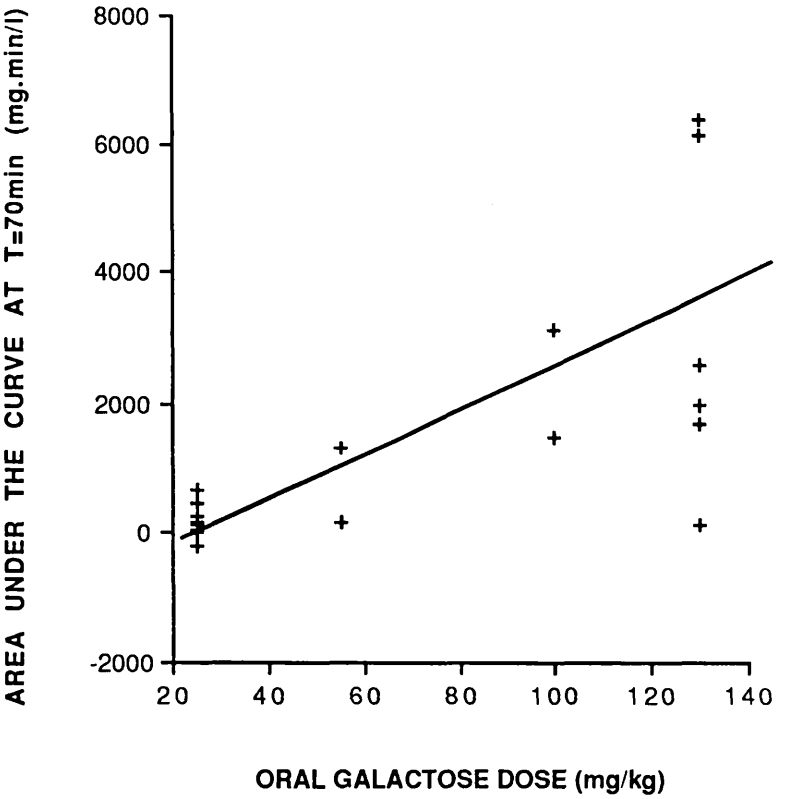


Figure 16

This shows decreasing areas under the curve with descending oral doses of galactose in sham operated rats. The relationship between area under the curve (AUC) and oral galactose dose (GD) is described by the linear regression equation AUC = 28.12GD-514.35. A correlation coefficient of 0.698 was found.

TABLE 12

Type	Dose	AUC_{total} Mean ± SD (mg.min/l)	$AUC_{t=70}$ Mean ± SD (mg.min/l)	$AUC/dose_{total}$ Mean ± SD (kg.min/l)	$AUC/dose_{t=70}$ Mean ± SD (kg.min/l)
SO (n=6)	130	4264 ± 3084	3159 ± 2538	32.8 ± 23.7	24.3 ± 19.5
SO (n=2)	100	3403 ± 1418	2325 ± 1153	34.3 ± 14.5	23.3 ± 11.5
SO (n=2)	50	1132 ± 1129	771 ± 813	20.6 ± 20.5	14.1 ± 14.8
SO (n=9)	25	434 ± 357	228 ± 263	17.4 ± 14.3	9.2 ± 10.5
PVL (n=7)	25	420 ± 229	257 ± 313	16.9 ± 9.1	10.3 ± 12.5

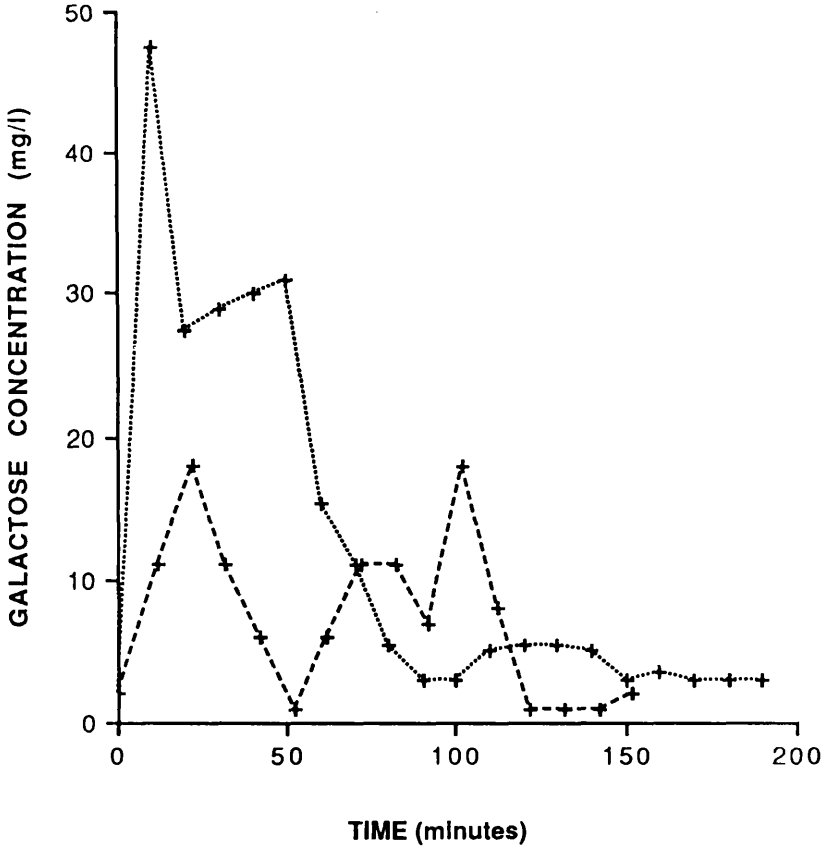
AUC_{total} = Total area under the galactose appearance curve

$AUC_{t=70}$ = Area under the galactose appearance curve from 0 to 70 minutes

$AUC/dose_{total}$ = Total systemic availability of galactose after an oral dose

$AUC/dose_{t=70}$ = Systemic availability of galactose from 0 to 70 minutes after an oral dose

This table shows the mean and standard deviation of the mean of AUC_{total} and other calculated expressions at various oral galactose doses in sham operated rats and in portal vein ligated rats at an oral dose of 25 mg/kg. No significant difference was found between sham operated and portal vein ligated rats in AUC_{total} ($p=0.906$), $AUC_{t=70min}$ ($p=0.751$), $AUC/dose_{total}$ ($p=0.922$), and $AUC/dose_{t=70min}$ ($p=0.757$) at an orál galactose dose of 25 mg/kg.



..... GALACTOSE CONCENTRATION TIME CURVE IN SHAM OPERATED RAT 1.

------- GALACTOSE CONCENTRATION TIME CURVE IN SHAM OPERATED RAT 2.

Figure 17

This shows galactose concentration time curves over a 2hr 30min time period in two sham operated rats following oral administration of a 130 mg/Kg dose of galactose.

TABLE 13

Dose (mg/kg)	Duration of sample (mins)	AUC total (mg.min/l)	AUC t=70 (mg.min/l)	CGmax 1 (mg/l)	CGmax 2 (mg/l)	Tpk 1 (min)	Tpk 2 (min)	AUC/dose total (kg.min/l)	AUC/dose t=70 (kg.min/l)
25	70	380	380	17	-	30	-	15.2	15.2
25	70	890	890	70	-	10	-	35.6	35.6
25(i)	150	505	255	12	-	50	-	20.2	10.2
25	150	320	90	5	-	(a)		12.8	3.6
25	150	380	190	5	-	(a)		15.2	7.6
25	150	275	-45	2	5	(a)		11.5	-1.8
25	110	190	40	4	-	(a)		7.6	1.6

AUC_{total} = Total area under the galactose appearance curve
$AUC_{t=70}$ = Area under the galactose appearance curve from 0 to 70 minutes
CGmax = Maximum blood galactose concentration after oral dose
Tpk = Time of blood galactose appearance peak
$AUC/dose_{total}$ = Total systemic availability of galactose after an oral dose
$AUC/dose_{t=70}$ = Systemic availability of galactose from 0 to 70 minutes after an oral dose
(i) = Food present in stomach
(a) = No definable appearance peak

Individual values of AUC_{total} and other expressions have been calculated in portal vein ligated rats after a 25 mg/kg oral dose of galactose.

DISCUSSION

Experiments in sham operated rats suggest that an oral dose of no more than 25 mg/kg is required to be sure of avoiding significant systemic breakthrough of galactose. However, in contrast to the experiments in patients described in Chapter 4, no increase in systemic availability was observed when the same dose was given to portal vein ligated rats. The erratic appearance curve seen in some animals suggests that absorption of galactose from the gastrointestinal tract in rats may be similarly erratic, delayed or incomplete, and this source of variability could mask differences between portal vein ligated and sham operated groups.

It is unlikely that oral galactose would enter the portal circulation via a different route from the microspheres with which the shunting characteristics of the model were established. Microspheres enter the portal circulation following injection into the ileocolic cannula at a point distal to the superior mesenteric vein. Galactose, in turn is absorbed from the small intestine and enters the portal vein via the superior mesenteric vein. The main pattern of collateral development in rats with a prehepatic portal vein stenosis is through splenorenal collaterals and retrograde filling of the inferior mesenteric vein

(Halvorsen and Myking, 1974; Bengmark et al, 1976). Both microshperes and galactose should encounter these collaterals upon entry into the portal vein and be quantitatively diverted into the systemic circulation. However, because of the failure to demonstrate a significant difference in the systemic availability of galactose between portal vein ligated and sham operated rats, it would seem possible that galactose may not be subjected, for whatever reasons, to the same degree of portasystemic shunting as that of the microspheres.

A third possibility for the failure to demonstrate a difference in the systemic availability of galactose between sham operated and portal vein ligated rats is that the galactose concentration threshold established in these experiments, above which extraction by the normal liver is incomplete, is so low in the rat that it gives rise to blood concentrations below the limits of sensitivity of the assay even when shunting is present. As discussed in chapter 3, resolution at blood galactose concentrations of 10 mg/l and below, is reduced as one reaches the limits of assay sensitivity. Such blood galactose concentrations are common after an oral dose of 25 mg/kg of galactose.

In the final sections of this chapter we examine the first two of these possibilities, and in the following chapter we address the other one.

SECTION B. CARBON-14 LABELLED GALACTOSE

INTRODUCTION

This section examines the idea that erratic and/or incomplete absorption of galactose from the gut maybe part responsible for the non-significant difference in systemic availability of oral galactose between sham operated and portal vein ligated rats. By measuring the amount of radioactivity present in various sections of the gastrointestinal tract following oral administration of carbon-14 labelled galactose in sham operated, and portal vein ligated animals, the percent retention of galactose in each section of the gastrointestinal tract can be calculated and a picture formed of the distribution and retention of galactose in the gut. The techniques used and results are described in this section.

MATERIALS AND METHODS

Animals were prepared as described in Chapter 5. Animals weighting 354.5 ± 46.6 grams (mean ± SD) were used in all experiments. A solution was prepared containing 4 mg/ml unlabelled galactose and 0.04 MBq/ml carbon-14 labelled galactose, dissolved in distilled water. A dose containing 25 mg/kg body weight of unlabelled galactose was flushed into the stomach of the rat, twenty minutes after a 2ml water gavage. Arterial blood samples were collected every 10 minutes, and samples analysed, after deproteinisation, as described in Chapter 3. Prior to orogastric administration of galactose an arterial blood sample was collected to determine basal galactose concentrations.

At the end of the experimental period, approximately 2 hours, the rat was sacrificed by opening the chest, an incision was then made in the abdomen and the stomach together with contents and the intestines were removed. The intestines were divided into the duodenum, the remainder of the small bowel, and the large bowel. Each section was sliced individually into approximately 1 cm long segments, the stomach was dealt with similarly, after the removal of its contents. Tissue samples and stomach contents were then placed in previously weighed liquid scintillation counting vials

and digested by the addition of 10ml 10M concentrated sodium hydroxide for two days in an oven at a temperature of approximately 60 to 80 degrees C. When digestion was complete each vial was weighed once again, and 0.5ml of the digested tissue removed for counting of radioactivity. The vial was then re-weighed again to determine the tissue sample weight.

The 0.5ml digested tissue sample was then bleached by the addition of 0.5ml 30% hydrogen peroxide for 1 hour. 10 ml of liquid scintillant, Optiphase "Safe" (LKB) was added, and the sample counted in a Packard Tri-Carb 2660 liquid scintillation counter with automatic quench correction. The counter was regularly calibrated with a set of quenched carbon-14 standards. A 0.25ml sample of the 4mg/ml original stock solution was also counted for radioactivity and the following calculations made;

Tissue calculations

1. Total tissue solution weight

The total weight of the digested stomach, duodenum and the small and large bowel (including solvent) were individually calculated.

Total tissue solution weight (grams)

= Weight of vial after digestion of tissue
weight of the empty vial (g)

2. Radioactivity in tissue sample

Radioactivity per gram (DPM/g)

$$= \frac{\text{Radioactivity in 0.5ml digested tissue sample (DPM)}}{\text{Weight of 0.5 ml sample (g)}}$$

3. Radioactivity in total tissue

Radioactivity in total tissue (DPM)

= Radioactivity in tissue sample (DPM/g)
multiplied by total tissue weight (g)

4. Radioactivity administered

Radioactivity administered (DPM)

= Radioactivity in 0.25ml stock solution (DPM) multiplied by amount of oral galactose given (ml) multiplied by 4

5. Percentage radioactivity in tissue

Percentage radioactivity in tissue (%DPM)

$$= \frac{\text{Radioactivity in total tissue (DPM)}}{\text{Administered radioactivity (DPM)}} \times 100$$

Blood samples collected from the right femoral artery cannula were also counted for carbon-14 radioactivity after deproteinization as described in Chapter 3. 0.2ml of the resultant supernatant was counted by placing in a liquid scintillation counting vial with 10 ml of liquid scintillant, Optiphase "Safe" (LKB), and then counted in a Packard Tri-Carb 2660 liquid scintillation counter, as previously described.

Statistical Analysis

The statistical significance of observed differences between sham operated and portal vein ligated animals was tested by the Student's t-test.

RESULTS

The percentage of carbon-14 labelled galactose retained in various sections of the gastrointestinal tract in three sham operated and four portal vein ligated animals after orogastric administration of 25 mg/kg of galactose is shown in Table 14. The extent of retention two hours after the initial oral dose in sham operated rats ranged from 3.62 to 15.29 percent, with a mean of 10.47 $\pm$ 6.09% (SD), and in portal vein ligated rats ranged from 15.15 to 34.64 percent, with a mean of 21.06 $\pm$ 9.16% (SD). This difference was not statistically significant (p=0.147).

The greatest individual percent retention of carbon-14 galactose occurred in the gastric fluid of a portal hypertensive rat (28.50%), however in both control and portal vein ligated rats the greatest mean percent retention of carbon-14 galactose was found in the small intestine and the stomach. Overall the percent retention in various sections of the gastrointestinal tract varied greatly from animal to animal (Table 14).

In three sham operated rats and one portal vein ligated rat systemic blood galactose concentrations were measured, and the total area under the galactose apparance curve (AUC total), area under the galactose appearance curve from 0 to 70 minutes (AUC t=70min), AUC/dose total, AUC/dose t=70min calculated and

tabulated against corresponding percent retention of carbon-14 galactose in the gastrointestinal tract following oral administration of 25 mg/kg of galactose (Table 15). The largest area under the curve was associated with the smallest amount of percent carbon-14 retained in the gastrointestinal tract, and a trend towards increasing retention with decreasing area under the curve was seen in this small group of rats (Figure 18). The relationship between total area under the galactose appearance curve (AUC) and percent retained carbon-14 radioactivity (RR) in the gastrointestinal tract is given by the linear regression equation AUC = 489.2 - 34.3RR. A correlation coefficient of 0.911 was found (Figure 18).

In all experiments collected blood samples were measured for carbon-14 radioactivity. The measured radioactivity was converted into equivalent galactose concentration (mg/l) by dividing the concentration of carbon-14 (MBq/l) by the specific activity of the original galactose solution (MBq/mg). Figure 19 shows a galactose concentration time curve and its corresponding carbon-14 radioactivity curve translated into equivalent mg/l from a portal vein ligated rat following oral administration of 25 mg/kg of galactose. Both graphs initially follow a similar pattern as systemic blood galactose concentrations increase following oral galactose administration. Systemic blood

galactose concentrations then decline with time as galactose is removed from blood by the liver, however equivalent galactose concentration remain elevated at an almost steady state. This elevate state can be attributed to circulating carbon-14 labelled metabolites of galactose which are released into blood as galactose is metabolised in the liver.

TABLE 14

PERCENT DPM

PROCEDURE	GASTRIC INTESTINAL SEGMENTS					
	GASTRIC FLUID	STOMACH	DUODENUM	SMALL INTESTINE	LARGE	TOTAL AMOUNT RETAINED
SO	(a)	1.28	(b)	1.28	1.06	3.62
SO	(a)	2.29	0.80	4.93	4.48	12.50
SO	(a)	7.47	0.78	7.00	0.04	15.29
Mean		3.68	0.79	4.40	1.86	10.47
SD		3.32	0.01	2.90	2.32	6.09
PVL	(a)	12.23	4.78	1.10	0.33	18.44
PVL	28.50	1.73	3.69	0.62	0.10	34.64
PVL	2.97	2.45	3.05	6.59	0.09	15.15
PVL	1.51	1.00	0.29	12.34	0.87	16.01
Mean	10.99	4.35	2.95	5.16	0.35	21.06
SD	15.18	5.28	1.91	5.50	0.37	9.16

(a) = Gastric fluid counts included with stomachs
(b) = Duodenum counts included with small bowel

A comparison of the percentage of carbon-14 labelled galactose retained in various sections of the gastrointestinal tract in both sham operated and portal vein ligated rats after orogastric administration. The mean percentage retention of galactose in the gastrointestinal tract in all 7 animals was 16.52 $\pm$ 8.07 ($\pm$ SD).

TABLE 15

Procedure	Dose (mg/kg)	AUC total (mg.min/l)	AUC t=70 (mg.min/l)	AUC/dose total (kg.min/l)	AUC/dose t=70 (kg.min/l)	Total amount retained (%)
SO	25	340	245	13.6	10.2	3.62
SO	25	165	-180	6.6	-7.2	12.50
SO	25	-115	25	-4.6	1.0	15.29
PVL	25	190	40	7.6	1.6	18.44

AUC_{total} = Total area under the galactose appearance curve

$AUC_{t=70}$ = Area under the galactose appearance curve from 0 to 70 minutes

$AUC/dose_{total}$ = Total systemic availability of galactose after an oral dose

$AUC/dose_{t=70}$ = Systemic availability of galactose from 0 to 70 minutes after an oral dose

This shows the total area under the galactose appearance curve from concentration time graphs and other calculated expressions together with the corresponding percentage of carbon-14 galactose retained in the gastrointestinal tract following oral administration of 25 mg/kg galactose in control and portal hypertensive rats.

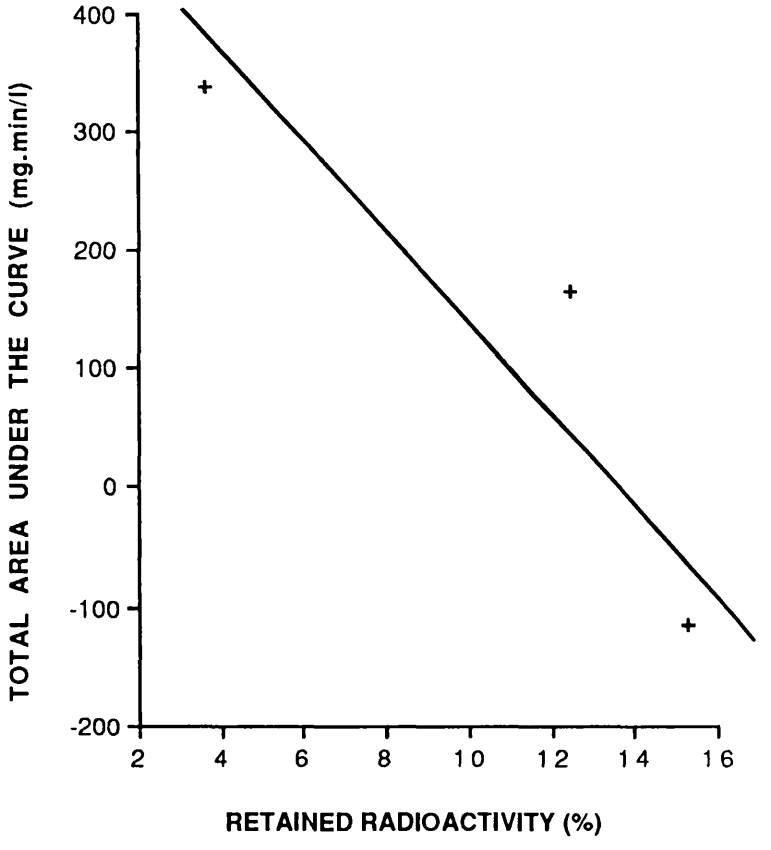


Figure 18

This shows the negative relationship between total area under the curve (AUC) and percent retained C-14 radioactivity (RR) in the gastro-intestinal tract in a small group of sham operated rats. The relationship is described by the linear regression equation AUC = 489.2-34.3RR.A correlation coefficient of 0.911 was found.

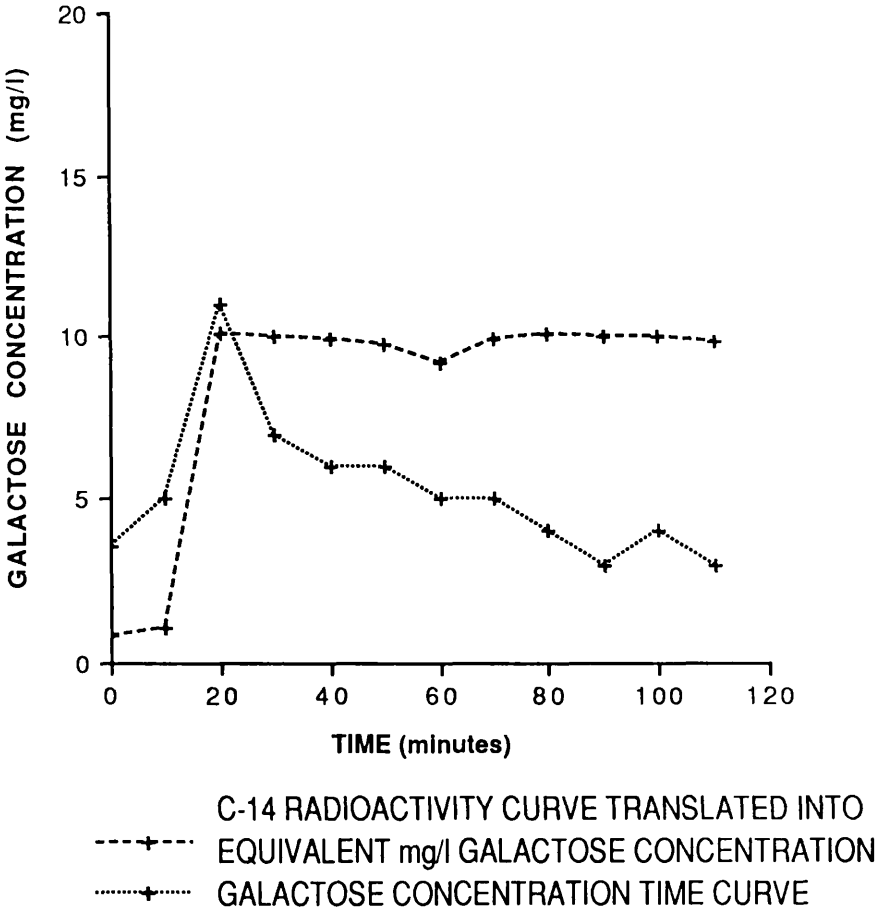


Figure 19

This shows a galactose concentration time curve and its corresponding Carbon-14 radioactivity curve translated into equivalent mg/l galactose concentration after a 25 mg/Kg oral dose of galactose in a portal vein ligated rat. As blood galactose concentration declines, equivalent mg/l galactose concentration remains at an almost constant level. This is attributed to circulating ^{14}C-labelled galactose metabolites.

DISCUSSION

The purpose of these experiments were to examine galactose retention in the gastrointestinal tract and its possible relationship in the failure to demonstrate a significant difference in systemic availability between sham operated and portal vein ligated rats as shown in the previous section. Experiments in both sham operated and portal vein ligated rats have shown absorption of galactose from the gastrointestinal tract to be incomplete two hours after the initial oral dose. Galactose retention also varied widely from animal to animal, with a trend towards a reduction in the systemic availability of galactose with increasing retention. These observations would explain the erratic, and delayed galactose appearance curves seen in some animals in the previous section, and this source of variability could indeed mask differences in the systemic availability of galactose between portal vein ligated and sham operated groups.

The erratic and incomplete absorption of galactose from the gastrointestinal tract could be attributed to the presence of food in the gastrointestinal tract, and/or the effect of anaesthesia on gastric emptying. It is unlikely that galactose retention alone could account for the failure to demonstrate a significant difference in systemic availability between portal vein ligated

and control animals; on average less than 20 percent of an oral galactose dose was retained in the gastrointestinal tract. An alternative possibility, as discussed in the previous section, is that galactose upon entering the portal vein is not subjected to the same degree of portasystemic shunting, for reasons that are at present unclear, as that of injected microspheres with which the characteristics of the model were established. In the final part of this chapter we infuse galactose directly into the portal vein. By this route galactose not only follows the same path as injected microspheres, but overcomes the problems of gastric retention.

SECTION C. SINGLE INFUSION OF GALACTOSE INTO THE PORTAL VEIN

INTRODUCTION

Experiments up to this point have examined the systemic availability of galactose following oral administration of various doses of galactose, and the percent retention of galactose in various sections of the gastrointestinal tract in both control and portal hypertensive rats. In the previous sections we suggested that the failure to demonstrate a significant difference in systemic availability of oral galactose between portal hypertensive and control rats stemmed from erratic and incomplete absorption of galactose from the gut and a possibility that galactose was not subjected to the same degree of portasystemic shunting as microspheres upon entry into the portal vein. In order to avoid these problems experiments were performed in which galactose was infused directly into the portal vein. By this route, the problems associated with absorption of galactose from the gut are circumvented, and also galactose follows the same path as injected microspheres, which in portal vein ligated animals are subjected to portasystemic shunting as discussed in Chapter 5.

This section describes the technique used and the results produced.

MATERIALS AND METHODS

Animals weighing 255.6 ± 29.43 grams (mean ± SD) were used in all experiments. Portal hypertension was produced using the portal vein ligation technique as described in Chapter 5. Animals were fasted for 24 hours before study in wire bottom stainless steel cages, and were allowed water ad libitum. Mean arterial pressure and arterial blood gases were measured as described in Chapter 5. The ileocolic branch of the portal vein was cannulated, and mean portal pressure measured prior to commencing the experiment as described in Chapter 5. Arterial blood samples were collected and analysed by galactose dehydrogenase enzyme assay as described in Chapter 3. Before infusion, an arterial blood sample was collected to determine basal galactose concentration.

D-galactose dissolved in distilled water at a concentration of 40 mg/ml was infused from a 5 ml glass syringe by a Braun infusion pump (B.Braun-Melsungen AG, West-Germany) at a rate of 0.9 ml/hr into the portal vein, giving galactose infusion rates in the range 128 mg/kg/hr to 176 mg/kg/hr. Timed arterial blood samples were collected every ten minutes, until 40 minutes when the infusion ended, and thereafter every five minutes for another seventy minutes.

Once arterial blood sampling was complete approximately

500,000 Cobalt-labelled microspheres (Co^{57}) were injected via the ileocolic cannula into the portal vein. Three minutes later the animal sacrificed by opening the chest, and the lungs, kidneys and liver were removed and placed in vials for counting radioactivity. The larger organs were dissected and placed in several vials for counting. The tissues were counted for radioactivity by a Gamma Scintilation counter (Packard Instruments, Auto-gamma 5000) and the magnitude of portasystemic shunting calculated as described in Chapter 5.

Analysis of the intraportal infusion

The forty minute intraportal infusion of galactose was analysed as if it represented an oral dose, completely absorbed from the gut at a uniform rate over the same period. The area under the galactose appearance curve was calculated as described in Section A, and the "oral" dose was equated with total infused dose.

Further, the slope of the blood galactose clearance curve after the termination of the infusion was used to provide additional information about systemic galactose clearance. Equation 2.6 of Chapter 2,

$$Ca(t)/dt = -(F/Vd).Ca(t) \quad \ldots\ldots\ldots\ldots\ldots\ldots 6.6$$

gives an expression relating the rate of disappearance of galactose from the blood to liver blood flow (F) and the distribution volume for galactose (Vd) under the assumptions that,

(a) the liver is the only organ of extraction

(b) hepatic extraction efficiency is 100 percent

(c) blood galactose is in continuous equilibrium with the total body pool and that elimination obeys first-order kinetics.

A more generalised form of this equation is,

$$dCa(t)/dt = -(K/Vd).Ca(t) \quad \ldots\ldots\ldots\ldots\ldots\ldots 6.7$$

where k is the systemic clearance, and no assumptions are made about the route of clearance. The solution to equation 6.7 is,

$$Ca(t) = Ca(0)\exp(-Kt/Vd) \quad \ldots\ldots\ldots\ldots\ldots\ldots 6.8$$

Where, Ca(0) = arterial concentration (Ca) at time zero.

or, taking logarithms,

$$\text{Log } (Ca(t)) = \text{Log } (Ca(0)) - Kt/Vd \quad \ldots\ldots\ldots\ldots 6.9$$

The factor K/Vd was calculated by linear regression analysis as the slope of the galactose clearance curve on a logarithmic scale.

AUC.K/dose.Vd calculation and Statistical analysis

The area under the appearance curve divided by its corresponding dose (AUC/dose) was calculated as described in section A. AUC/dose multiplied by clearance was also calculated. The latter term gives us an expression for the systemic availability of galactose related to the the volume of distribution (Vd). With reference to section A, equation 6.1 stated,

$$AUC/Q = SA/K = S + (1-S)(1-E)/K \quad \ldots\ldots\ldots\ldots 6.1$$

Where, SA = Systemic availability
K = Systemic galactose clearance
Q = Galactose dose

In this section, clearance is derived from the slope of the galactose clearance curve, and is described by K/Vd (equation 6.9). If equation 6.1 is multipied through by the term for systemic clearance (K/Vd), then

$$AUC.K/Q.Vd = SA/Vd = S + (1-S)(1-E)/Vd \quad .....6.10$$

gives an expression for systemic availability which is related to the distribution volume for galactose.
The statistical significance of observed differences between sham operated and portal vein ligated animals was tested by the Student's t-test.

RESULTS

Table 16 shows the area under the galactose appearance curve (AUC) in 15 individual sham operated and portal vein ligated rats at intraportal infusion rates ranging from 128 mg/kg/hr to 176 mg/kg/hr. The mean and standard error of the mean (SEM) was 3731 $\pm$ 462.24 mg.min/l and 3225.8 $\pm$ 359.26 mg.min/l in portal vein ligated and control animals respectively. No significant difference was found between both groups ($p = 0.395$).

The mean and standard error of the mean of galactose dose, systemic clearance, AUC/dose and AUC.K/dose.Vd in both groups of animals following a 40 minute intravenous infusion of galactose are shown in Table 17. The mean galactose dose $\pm$ SEM, 91.38 $\pm$ 3.42 mg/kg/hr given to portal vein ligated rats was greater than that received by sham operated rats, 81.56 $\pm$ 2.64 mg/kg/hr, this difference was statistically significant ($p = 0.03$).

Mean systemic clearance (K/Vd) $\pm$ SEM values of 0.092 $\pm$ 0.0091 min^{-1} and 0.094 $\pm$ 0.0071 min^{-1} in portal vein ligated and control animals respectively were not significantly different from each other ($p>0.05$). Figure 20 shows the galactose concentration time curve in a sham operated rat following a 40 minute intraportal infusion of galactose at an infusion rate

of 128 mg/kg/hr. The area under the galactose appearance curve was 1942 mg.min/l. The slope of the above galactose clearance curve (K/Vd) plotted on a logarithmic scale with regression line is shown in Figure 21. The gradient of the slope by linear regression analysis was 0.115 min^{-1}. The correlation coefficient was 0.99.

Mean AUC/dose and AUC.K/dose.Vd were not significantly different for the two groups ($p > 0.05$). The mean $\pm$ SEM for AUC/dose was 41.10 $\pm$ 5.14 kg.min/l and 39.91 $\pm$ 4.83 kg.min/l and for AUC.K/dose.Vd was 3.37 $\pm$ 0.31 kg/hr/l and 3.13 $\pm$ 0.29 kg/hr/l in portal vein ligated and sham operated rats respectively (Table 17).

The mean $\pm$ SD portasystemic shunting value calculated from injected microspheres in portal vein ligated rats was 56.38 $\pm$ 32.48 percent and 0.135 $\pm$ 0.238 percent in sham operated rats. Figure 22 shows the poor relationship between area under the galactose appearance curve (AUC) and portasystemic shunting (PSS) in portal vein ligated rats. The relationship is described by the linear regression equation AUC = 3155.76 + 3.28PSS, with a correlation coefficient of 0.070.

TABLE 16

Procedure	Area under the curve (AUC) (mg.min/l) PVL (n=15)	SO (n=15)
	1828	1942
	2442	4202
	2393	3054
	3978	3061
	4948	2422
	5396	3159
	3716	5293
	4828	3946
	5129	4567
	4399	5580
	1778	4401
	7408	1683
	1142	2345
	1639	1688
	4941	1044
MEAN	3731	3225.8
SEM	462.24	359.26

This shows individual areas under the galactose appearance curve (AUC) in both portal vein ligated (PVL) and sham operated (SO) animals after a forty minute intraportal infusion of galactose at infusion rates ranging from 128 to 176 mg/kg/hr. The mean $\pm$ SEM are also shown. No significant difference was found between PVL and SO rats ($p = 0.395$).

TABLE 17

	Procedure		
	PVL	SO	p value
	(Mean ± SEM)	(Mean ± SEM)	
Dose (mg/kg)	91.38 ± 3.42 (n=15)	81.56 ± 2.64 (n=15)	= 0.03
Clearance (min^{-1})	0.092 ± 0.0091 (n=12)	0.094 ± 0.0071 (n=10)	> 0.05
AUC/dose (kg.min/l)	41.10 ± 5.14 (n=15)	39.91 ± 4.83 (n=15)	> 0.05
AUC.K/dose.Vd (kg/hr/l)	3.37 ± 0.31 (n=12)	3.31 ± 0.29 (n=10)	> 0.05
	Mean ± SD	Mean ± SD	
PSS microspheres	56.38 ± 32.48 (n=13)	0.14 ± 0.24 (n=12)	< 0.05

This shows the mean ± SEM of galactose dose, and other calculated expressions, as defined in the following page, in both control (SO) and portal vein ligated animals (PVL) following an intraportal infusion of galactose. The difference in dose and percent portasystemic shunting between PVL and SO rats were the only expressions to be statistically significant ($p = 0.03$: $p < 0.05$).

The definitions of the calculated expressions in Table 17 are shown below;

Dose	=	Total amount of galactose given
Clearance	=	Slope of the galactose clearance curve (K/Vd)
AUC/dose	=	Systemic availability of galactose dependent upon clearance after intraportal infusion
AUC.K/dose.Vd	=	Systemic availability of galactose dependent upon volume of distribution after intraportal infusion
$PSS_{microspheres}$	=	Portasystemic shunting as measured by injected microspheres

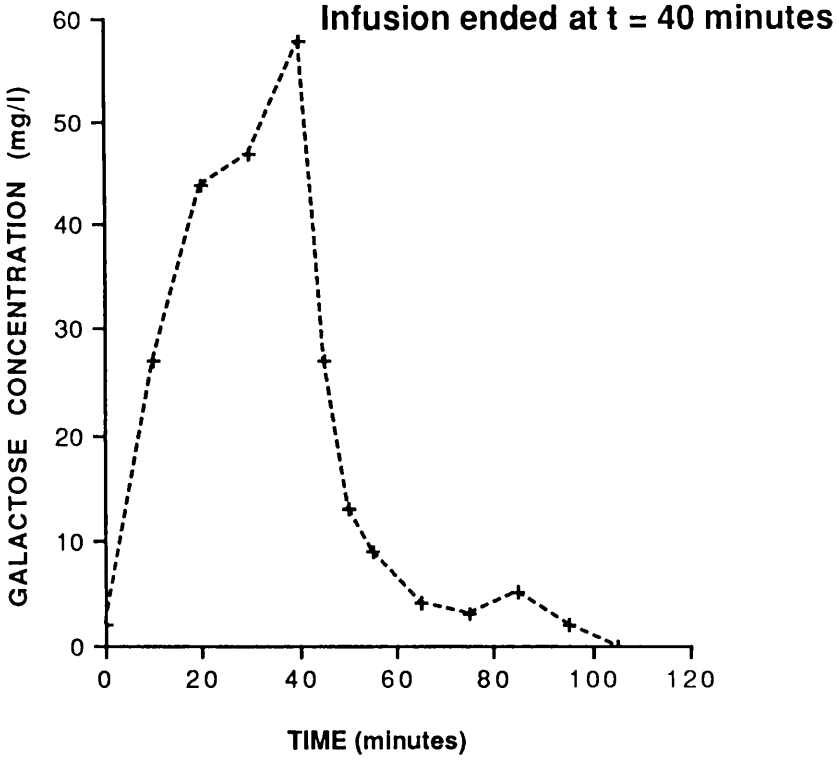


Figure 20

This shows the galactose concentration time curve in a sham operated rat following a 40 minute intraportal infusion of galactose at a rate of 128 mg/kg/hr. The area under the galactose appearance curve was 1942 mg.min/l.

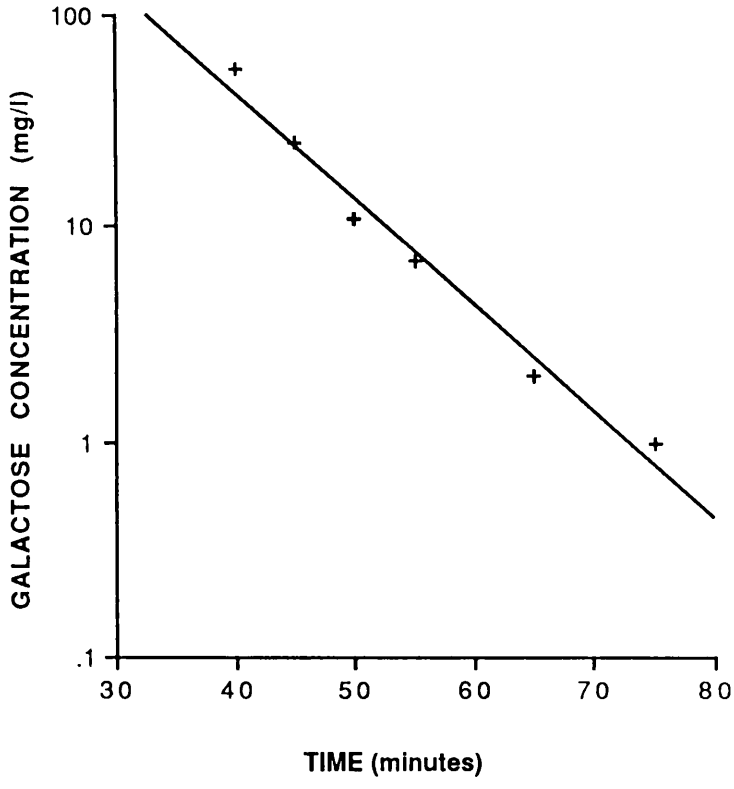


Figure 21

This shows the slope of the galactose clearance curve (K/vd) plotted on a logarithmic scale with regression line following a 40 minute intraportal infusion of galactose at a rate of 128mg/kg/hr in a sham operated rat. The gradient of the slope by linear regression analysis was 0.115 min-1. The correlation coefficient was 0.99.

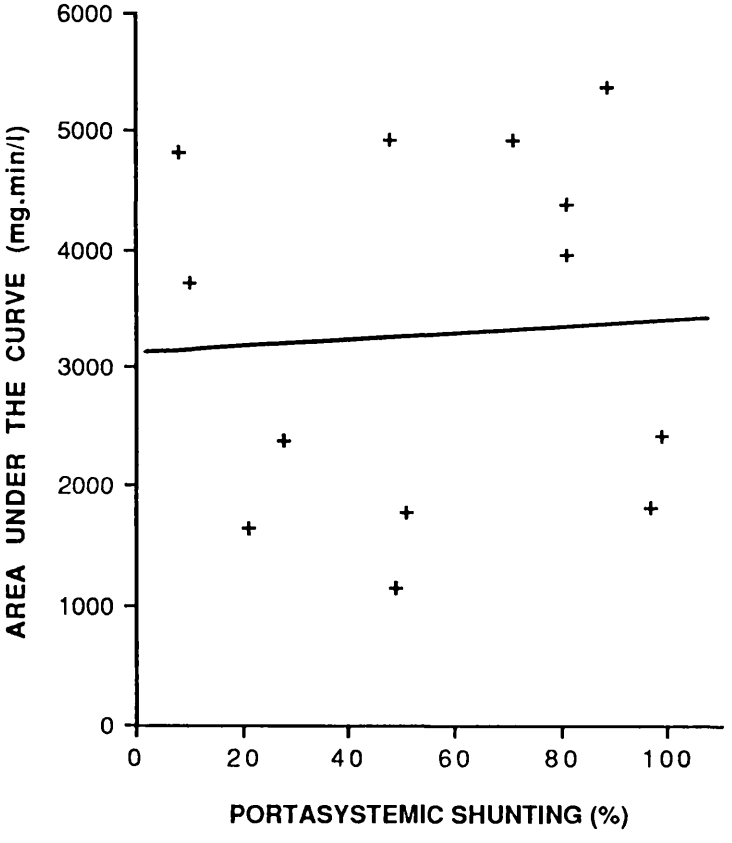


Figure 22

This shown the poor relationship between area under the curve (AUC) and portasystemic shunting (PSS) in portal vein ligated rats. The relationship is described by the linear regression equation AUC = 3155.76 + 3.28PSS. A correlation coefficient of 0.070 was found.

DISCUSSION

In these experiments, galactose was infused into the portal vein by the same route as injected microspheres, which showed a mean portasystemic shunting value of 56% in the portal vein ligated group. However, there was no difference in area under the galactose appearance curve (AUC) or AUC/dose. The failure to detect the expected greater systemic availability in portal vein ligated rats relative to sham operated rats in these experiments cannot be due to the difference in doses administered, as this was on average slightly higher in portal vein ligated rats. The possibility that differences between groups in total systemic galactose clearance (K) is compensating for the difference in shunting appears to be contradicted by the fact that the calculated parameters K/Vd and SA/Vd were similar in both groups. The calculation of these parameters is based on the some what doubtful assumption of instantaneous equilibration of galactose between blood and tissue during the disappearance phase of the curve. However it is inherently unlikely that K would be higher in the portal vein ligated that sham operated groups as would be necessary to compensate for differences in shunting. Portal vein ligated rats would be expected if anything to have lower liver blood flow and K values.

It is improbable that portal vein ligation caused any major alteration in the distribution volume (Vd). The most likely explanation for these results is that the extraction efficiency of the liver at the galactose concentrations achieved in these experiments is very low in the sham operated group. Thus a high degree of "functional" shunting in this group would have the same effect as anatomical and functional shunting combined in portal vein ligated rats.

CONCLUSIONS

Experiments have shown that erratic and incomplete absorption of galactose from the gastrointestinal tract is in part responsible for the failure to demonstrate a significant difference in the systemic availability of galactose between portal vein ligated and control rats following an oral dose of 25 mg/kg of galactose. This oral dose was established as the galactose concentration threshold above which extraction by the normal liver is incomplete.

The intraportal infusion of galactose further suggested that a similar systemic availability of galactose in both sham operated and portal vein ligated rats would arise if the hepatic extraction efficiency at the galactose concentrations encountered in this study, in sham operated, rats was very low.

In the following chapter we examine more closely the systemic availability of intraportally administered galactose in rats, and the dependency of hepatic extraction efficiency on circulating blood galactose concentrations.

CHAPTER SEVEN INTRAPORTAL AND INTRAVENOUS INFUSION OF GALACTOSE IN AN EXPERIMENTAL PREHEPATIC MODEL OF PORTAL HYPERTENSION

1. Introduction

2. Materials and methods

3 (a). Kinetic model and results

(b). Galactose clearance

(c). Extrahepatic galactose elimination

4. Discussion

INTRODUCTION

It has been hypothesised that the failure to demonstrate a significant difference in systemic availability of galactose between portal hypertensive and control rats, following oral administration of galactose, is a result of erratic and incomplete absorption from the gut, and a failure of galactose to "see" the major portacaval shunts as a result of its site of entry into the portal vein (Chapter 6). The results of the last section of chapter 6, however, not only suggested that the route of administration of galactose is not a factor in the failure to demonstrate a significant diference in systemic availablity of galactose between portal hypertensive and control rats, but also implied that the extraction efficiency of the liver was very low in the sham operated group at the doses used. This finding thus brought into question hepatic extraction efficiency in rats.

In this chapter we examine more closely the systemic availability of intraportally administered galactose in rats, and the dependence of hepatic extraction efficiency on circulating blood galactose concentrations. It has been shown that hepatic uptake of galactose by the hepatocytes follows Michaelis-Menten kinetics (Keiding et al, 1976; Keiding and Chiarantini, 1978). It has further been shown, by

Keiding and Bass (1983), that from the Michaelis-Menten equation, $V = VmaxCm/(Km+Cm)$ (Chapter 2) a formula for extraction efficiency (or rate) (E) can be developed where, $E = 1-exp[1-(Vmax-Inf)/FKm]$. In this equation the symbols Inf and F represent galactose infusion rate and liver blood flow respectively. This equation, however, has a relatively large number of unknown parameters and a simpler approach in the intact rat is to apply the Michaelis-Menten equation not to the hepatocyte alone, but to the whole organ. Thus V, and Vmax would represent the galactose elimination rate and its maximum value for the liver as a whole, and Km the concentration of galactose in the systemic circulation at which elimination is half maximal. Keiding (1973) has shown this to produce Km and Vmax values in reasonable approximation to those found for the hepatocyte. However, the 'Km' value for galactose in systemic blood is higher than 'Km' for hepatocytes owing to the decreasing galactose concentration as the blood flows through the liver (Keiding, 1973). This chapter considers explicitly the Michaelis-Menten equation in the context of experimental studies on galactose elimination kinetics in portal hypertension and focuses on the development of a theoretical model through which the kinetic parameters that may be influencing galactose clearance are clarified.

In the following experiments, galactose was infused by

both a portal and intravenous route in portal vein ligated and sham operated rats. At various galactose infusion concentrations, mean equlibrium blood galactose concentrations were measured and from resultant graphs the parameters Vmax and Km calculated. Expressions of hepatic extraction efficiency were derived and extraction efficiencies calculated. Mean galactose clearance was also measured at each intravenous infusion rate. Further, the magnitude of portasystemic shunting was calculated by both microsphere injection, as described in Chapter 5, and from shunting equations derived from the kinetic model. Each method was compared with the other.

Finally, a possible extrahepatic route of galactose elimination was examined by collecting and measuring, by enzyme assay and fluorescence spectrophotometry (Chapter 3), the concentration of galactose appearing in urine following completion of an experiement.

MATERIALS AND METHODS

Animals weighing 289.4 ± 41.7 grams were used. Portal hypertension was produced using the partial portal vein ligation technique as described in Chapter 5. Mean arterial pressure and arterial blood gases were measured as described in Chapter 5. Cannulation of the left femoral vein, was performed for the intravenous infusion, the ileocolic branch of the portal vein was cannulated for the portal infusion, and mean portal pressure measured prior to commencing the experiment as described in Chapter 5. Before the infusion, an arterial blood sample was collected to determine basal galactose concentration.

D-galactose dissolved, at a range of concentrations, in 5 ml of distilled water was infused from a 5 ml glass syringe by a Braun infusion pump (B.Braun-Melsungen AG, West-Germany) at a rate of 0.9 ml/hr into the ileocolic cannula and the intravenous cannula · sequentially in random order. Each infusion had a duration of one hour, and the same concentration of galactose was used for both infusions in a given experiment. Arterial blood sampling began after infusing for 30 minutes and continued thereafter every 10 minutes until 60 minutes. All arterial blood samples were analysed as described in Chapter 3.

Once arterial blood sampling was complete approximately

500,000 Cobalt-labelled microspheres (Co^{57}) were injected via the ileocolic cannula into the portal vein. After sacrifice, three minutes later the lungs, kidneys and liver were removed and placed in vials for counting radioactivity. The larger organs were dissected and placed in several vials for counting. The magnitude of portasystemic shunting was calculated as described in Chapter 5.

In some experiments, upon their completion, urine was was collected by suprapubic puncture of the bladder, and the concentration of galactose in the urine measured by enzyme assay as described in Chapter 5.

Design of the study

Portal vein ligated and sham operated animals were divided into four infusion groups, receiving a galactose infusion rate of either 25, 50, 75, and 100, mg/kg/hr. Additional experiments were performed with an intravenous infusion only at a rate of 125 mg/kg/hr.

KINETIC MODEL:

THE THEORY BEHIND THE GALACTOSE DUAL INFUSION TECHNIQUE AND RESULTS

With reference to the introduction, it has been shown that galactose is removed from the blood by the liver according to Michaelis-Menten kinetics (Keiding, 1973). Whole organ elimination of galactose is thus described by the Michaelis-Menten equation,

$$V = VmaxCm/(Km + Cm) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 7.1$$

where,

V = galactose elimination rate for liver
Vmax = maximum galactose elimination rate
Km = Michaelis-Menten constant - blood galactose concentration at half maximum elimination rate
Cm = mean concentration of galactose in blood entering liver by hepatic arterial (ha) and portal venous(pv) routes combined

As the model develops the superscripts ip and iv will be used when necessary to distinguish blood galactose concentrations for the two routes of infusion. Equations without these superscripts are valid for both infusions. All variables refer to the equilibrium state when nothing is changing with time.

Further assumptions that are made are,

1). The physiological parameters, i.e. Shunting (S), Blood flow (F), Vmax and Km are the same for both i.v

and i.p infusions. Infusion rate (I) is also the same for both the i.v and i.p infusions by design.

2). The liver is assumed to be the only significant organ of galactose elimination, so that the hepatic galactose elimination rate equals galactose infusion rate at equilibrium, V = I. It follows that V is also the same for both i.v and i.p infusions.

3). Galactose elimination follows Michaelis-Menten kinetics, with a rate that depends only on Cm and not arterial galactose concentration (Ca) or portal venous galactose concentration (Cp) individually. This is equivalent to assuming that arterial and portal blood mix before encountering hepatocytes.

The development of the kinetic model which will allow us to examine the variability of hepatic extraction efficiency stems primarily from equation 7.1 together with formulae derived from model definitions which will be described later.

INTRAVENOUS INFUSION OF GALACTOSE IN SHAM OPERATED RATS

Relationship between equilibrium blood galactose concentration and infusion rate

If we examine the intravenous infusion of galactose and assume that no galactose is extracted outside the liver, the concentration of galactose in blood entering the portal vein from the gut, and the mean concentration in blood supplying the liver (Cm), is the same as arterial concentration, ie.:

$$Cm^{iv} = Ca^{iv} = Cp^{iv} \quad \text{.....7.2}$$

and so equation 7.1 can be rewritten,

$$V = VmaxCa^{iv}/(Km + Ca^{iv}) \quad \text{.....7.3}$$

From Assumption 2 (V = I), equation 7.3 becomes,

$$I = VmaxCa^{iv}/(Km + Ca^{iv}) \quad \text{.....7.4}$$

Experimental studies carried out in which equilibrium blood galactose concentrations at various infusion doses were measured, produced a hyperbola plot, from which, by non-linear regression analysis (Statgraphics statistical graphic system), the parameters Vmax and Km were estimated. Figure 23 shows equilibrium systemic galactose concentrations over 15 minutes following an intraportal and intravenous infusion of galactose at an

infusion rate of 100 mg/kg/hr. The gradient by linear regression analysis on data from 45 to 60 minutes of the intravenous infusion expressed as percent of the mean systemic galactose concentration was 0.17 ± 0.41 (SD) percent/min, and the range was -0.77 to +1.70. The increasing trend in systemic galactose concentrations at an intravenous infusion rate of 100 mg/kg/hr is due to the fact that at this infusion rate we are on that part of the hyperbola curve which is approaching the maximum galactose elimination rate (Vmax) and saturation kinetics.

Figure 24 shows the hyperbola plot in sham operated rats. By curve fitting Vmax was found to equal 177.2 ± 14.2 mg/kg/hr and Km to equal 43.5 ± 7.7 mg/l (mean ± SEM).

A more widely used approach to the determination of Vmax and Km from data of this kind is to rearrange equation 7.4 in linear form. Of the various forms introduced, the "Lineweaver-Burk plot" is the more frequently applied (Engel, 1981). Taking reciprocals of both sides of equation 7.4,

$$1/I = Km + Ca^{iv}/Vmax.Ca^{iv}$$

$$= Km/Vmax.Ca^{iv} + Ca^{iv}/Vmax.Ca^{iv}$$

$$= Km/Vmax.Ca^{iv} + 1/Vmax \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.5$$

or by rearranging,

$$1/Ca^{iv} = Vmax/Km.I - 1/Km \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.6$$

Figure 25 shows the plot of $1/Ca^{iv}$ against 1/I to be linear, with a slope of Vmax/Km and an intercept on the ordinate of -1/Km. The estimated values of Km and Vmax, derived by linear regression analysis (Statgraphics, statistical graphic system) were 89.2 ± 32.7 mg/l and 280 ± 121 mg/kg/hr (mean ± SEM). The standard errors of these values are much larger than those derived from the non-linear curve fitting procedure, and they are very heavily weighted by systemic galactose concentrations from the 25 mg/kg/hr intravenous infusion, which are subject to relatively high percent errors of measurement. The Vmax and Km values of 177.2 mg/kg/hr and 43.5 mg/l respectively, derived from non-linear regression analysis, are deemed more accurate and are thus the preferred values.

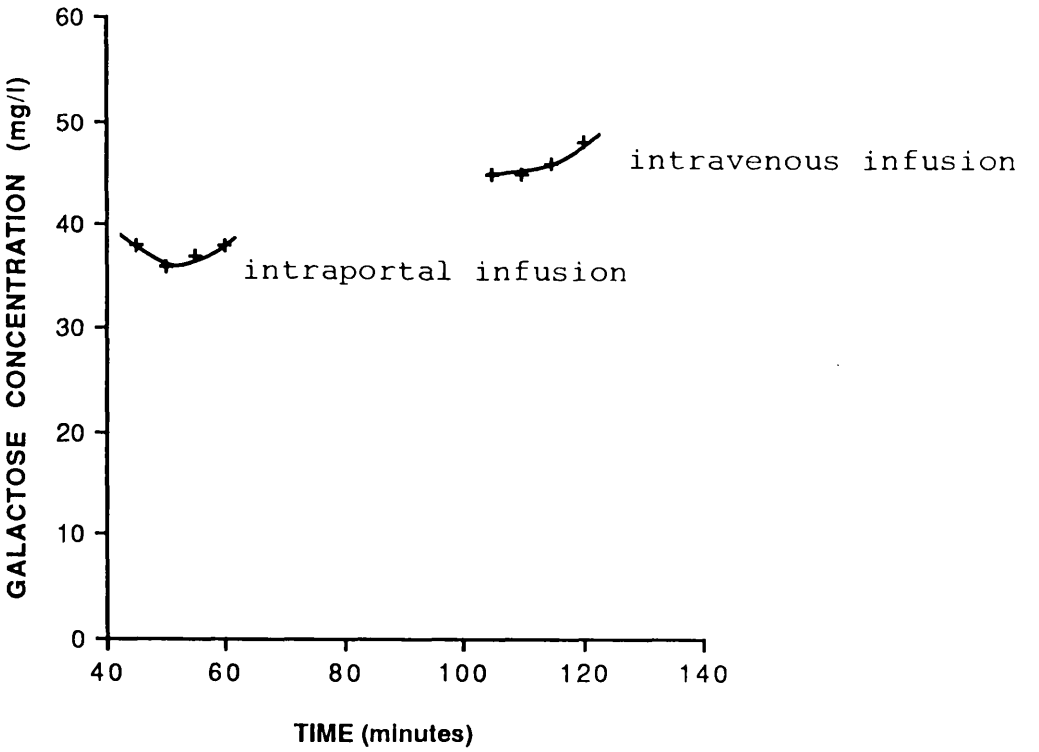


Figure 23

This shows equilibrium systemic galactose concentrations over 15 minutes following an intraportal and intravenous infusion of galactose at a rate of 100 mg/Kg/hr. The gradient of the line by linear regression analysis on data from the intravenous infusion of galactose expressed as % of mean galactose concentration was 0.17%/min ± 0.41 (SD). The range was -0.77 to +1.7.

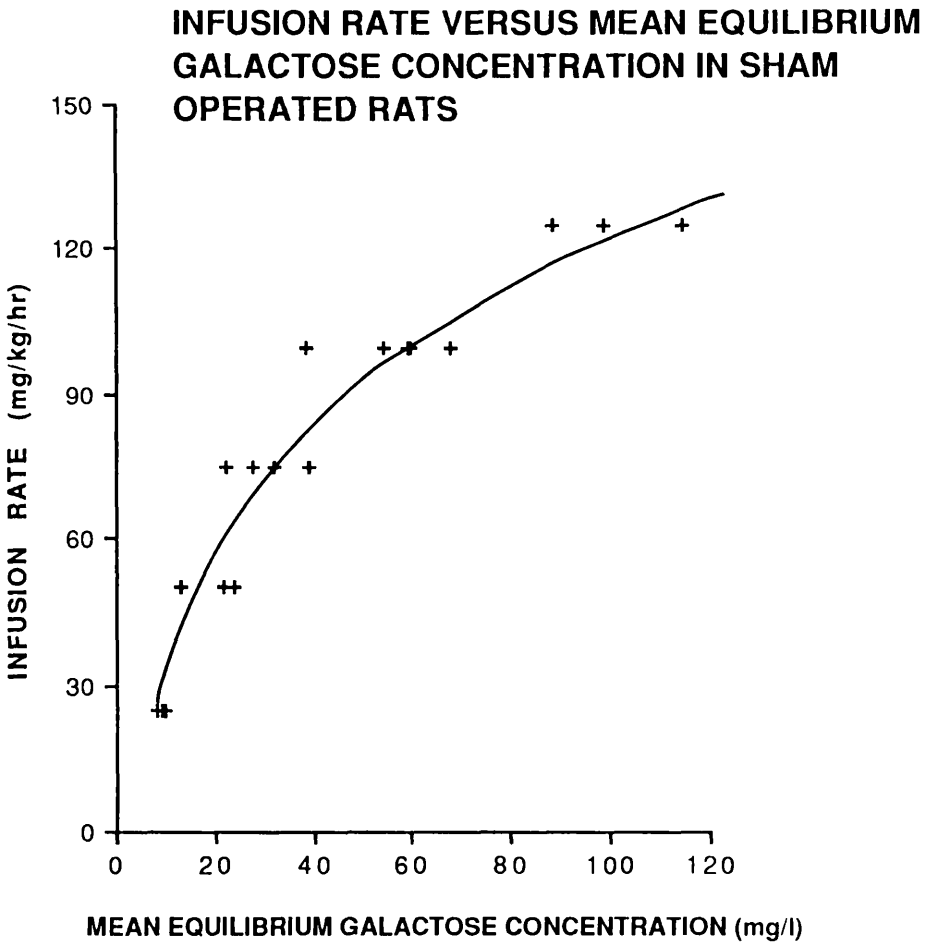


Figure 24

This shows the relationship between mean equilibrium blood galactose concentration at various intravenous infusion rates in sham operated rats. By non-linear regression analysis the parameters Vmax and Km of the curve can be estimated. In this case Vmax was 177.2 ± 14.2 mg/kg/hr, and Km was 43.5 ± 7.7 mg/l, (Mean ± SE).

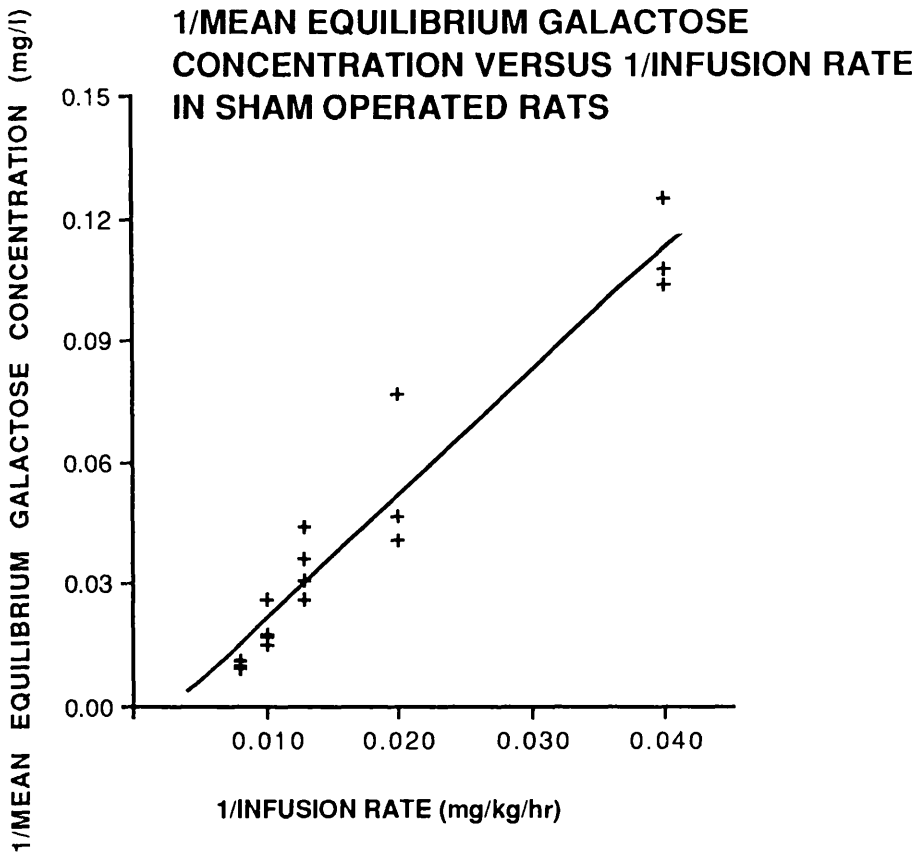


Figure 25

The "Lineweaver-Burk plot" gives a linear relationship between mean equilibrium blood galactose concentrations and intravenous infusion rates, and allows Vmax, and Km to be estimated from the slope of the line and its intercept on the ordinate respectively. In this case the mean ± SE of Vmax was 280 ± 121 mg/kg/hr and Km was 89.2 ± 32.7 mg/l in sham operated rats.

Relationship between expressions of hepatic extraction efficiency

One of the aims of this chapter was to examine the variability of hepatic extraction efficiency. With knowledge of the values for Vmax and Km, together with a number of assumptions and formula derived from model definitions, two expressions for extraction efficiency of the liver for galactose (E) can be derived. By definition, extraction efficiency (E) is the rate of hepatic extraction (V) divided by the rate of delivery of galactose, the latter being given by the concentration of galactose in blood (Cm) multiplied by total liver blood flow (F_l),

ie. $E = V/F_lCm$ 7.7

or from equation 7.1,

$E = Vmax/F_l(Km + Cm)$ 7.8

When Cm is very low (ie. negligible compared with Km), equation 7.8 becomes;

$E = Vmax/F_lKm$ 7.9

It is normally assumed at sufficiently low blood concentrations, hepatic extraction of galactose is complete (ie. E = 1). However this assumption can be tested, thus at low Cm, extraction is represented by

Eo, and equation 7.9 shows that;

$$Vmax/F_1Km = Eo \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 7.10$$

Hence, by rearranging and substituting equation 7.10 into 7.8,

$$E = Eo.Km/(Km + Cm) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots 7.11$$

For the intravenous infusion, equation 7.11 becomes,

$$E = Eo.Km/(Km + Ca^{iv}) \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.12$$

Another expression can be derived by inserting equation 7.10 into equation 7.1, and solving for Cm:

$$Cm = V.Km/(F1.Eo.Km - V)$$
$$= V.Km/(Vmax - V) \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.13$$

Inserting equation and 7.13 into equation 7.11,

$$E = [1 - (V/Vmax)].Eo \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.14$$

These two expressions for extraction efficiency, which for brevity we refer to as E_1 and E_2, are compared in Figure 26, ignoring the common unknown factor Eo. Each equation is compared as if each rat has the group mean values of Km and Vmax. The extraction term in the equation $E_2 = Eo.Km/(Km + Ca^{iv})$ is dependent upon individual equilibrium blood galactose concentration measurements in rats, whereas in equation $E_1 = [1 -$

(V/Vmax)].Eo extraction is independent of any individual rat measurements. By linear regression analysis the relationship between E_2 and E_1 is given by the equation $E_2 = 0.040 + 0.923E_1$, with a correlation coefficient of 0.970. The standard error (SE) of the intercept and the slope were 0.034 and 0.057 respectively. This result shows that the linear relationship is close to the line of identity. This reinforces the validity of the theory and our approach to applying Michaelis-Menten kinetics to the whole organ.

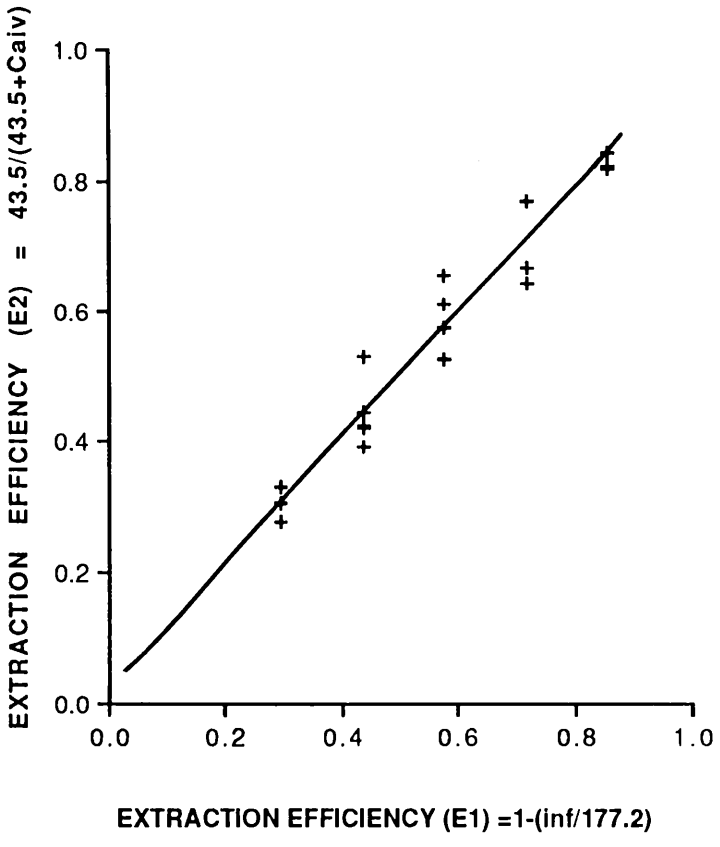


Figure 26

This shows the good relationship between two extraction efficiency expressions, 1-(inf/177.2), denoted by E_1 and 43.5/(43.5+ Ca^{iv}) denoted by E_2, in sham operated rats following intravenous infusions of galactose. The relationship is described by the linear regression equation $E_2 = 0.040 + 0.923E_1$. A correlation coefficient of 0.970 was found.

INTRAPORTAL INFUSION IN SHAM OPERATED RATS

If we now examine the intraportal infusion of galactose in sham operated rats, we can derive a third expression for extraction efficiency (E_3). This expression can then be compared with the two previous expressions of extraction (E_1 and E_2), derived from the intravenous infusion of galactose, in order to test the assumptions and self consistency of the theory.

The concentration of recirculating galactose in blood entering the portal vein from the gut is equal to arterial concentration, as with the i.v. infusion. However, this concentration is increased by the galactose being directly infused into the portal vein. The increase in concentration is given by the rate of galactose infusion (I) divided by the volume of blood flowing in the portal vein per unit of time (Fpv), ie. I/Fpv. Hence, total galactose concentration in the portal vein (Cp^{ip}) is given by,

$$Cp^{ip} = Ca^{ip} + I/Fpv \quad \text{.....7.15}$$

From Assumption 2 (V = I), and by rearranging the extraction efficiency equation 7.7,

$$I = E.F_l.Cm \quad \text{.....7.16}$$

When arterial galactose concentration (Ca) and portal venous galactose concentration (Cp) are different Cm is

given by their average weighted according to flow in artery and portal vein, ie. equation 7.17,

$$Cm = (FhaCa + FhpCp)/F_l \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.17$$

where,

Fhp = hepatic portal flow
Fha = hepatic arterial flow

Substituting equation 7.17 into equation 7.16,

$$I = E(FhaCa^{ip} + FhpCp^{ip}) \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.18$$

Substituting equation 7.15 into equation 7.18,

$$I = E(FhaCa^{ip} + Fhp(Ca^{ip} + I/Fpv)) \quad \ldots\ldots\ldots\ldots 7.19$$

Total liver blood flow equals the sum of hepatic arterial flow and the hepatic component of portal venous flow, i.e. $F_l = Fha + Fhp$ and by substituting into equation 7.19,

$$I = E(F_lCa^{ip} + IFhp/Fpv) \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.20$$

In sham operated rats portasystemic shunting is zero and hepatic portal flow (Fhp) equals portal venous inflow (Fpv), and equation 7.20 becomes,

$$I = E(F_lCa^{ip} + I) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 7.21$$

To eliminate the unknown variable F_l from equation 7.21, we must draw on the results of the intravenous infusion. We first note that E must have the same

value for both infusions. This follows from the fact that the derivation of equations 7.10, 7.13, and 7.14 does not depend on the route of administration of galactose, and these expressions for E and Cm are dependent only on variables which are assumed to remain constant. We may express equation 7.7 as,

$$E = I/F_1Ca^{iv} \quad \text{.....7.22}$$

Substituting into equation 7.21 and rearranging,

$$E = 1 - Ca^{ip}/Ca^{iv} \quad \text{.....7.23}$$

This equation gives us our third extraction efficiency expression (E_3), and unlike the other two hepatic extraction efficiency expressions it is dependent only on data derived from individual animals. By comparing this expression of extraction efficiency with the two previous derived expressions the relationship between the terms can be examined. The expected relationships are $E_3 = Eo.(1 - I/Vmax)$, and $E_3 = Eo.Km/(Km + Ca^{iv})$. E_3 is plotted against 1-I/Vmax and $Km/(Km+Ca^{iv})$ in Figures 27 and 28. The regression line in both cases does not differ significantly from the line of identity, and from its gradient the estimated value for Eo is 1.00 $\pm$ 0.22 ($\pm$SEM) (Figure 27) and 0.96 $\pm$ 0.26 ($\pm$SEM) (Figure 28). The results of these comparisons therefore suggest that the hepatic extraction efficiency for galactose at very low

concentrations is effectively 100%. The extraction efficiency expression (Eo) can thus be dropped from the equations. The good agreement between mean values for extraction efficiency (E) derived from the intravenous and intraportal infusions in individual animals (E_3), and the values derived from the collective intravenous data alone (E_1 and E_2), shown in Table 18, suggests that the assumptions underlying this analysis are sound. Thus the relationship between infusion rate, Ca^{iv}, and Ca^{ip} is consistent with the theory. Individual variations in Km and Vmax values would account for the scatter of the data points in Figures 26, 27 and 28 about the regression line.

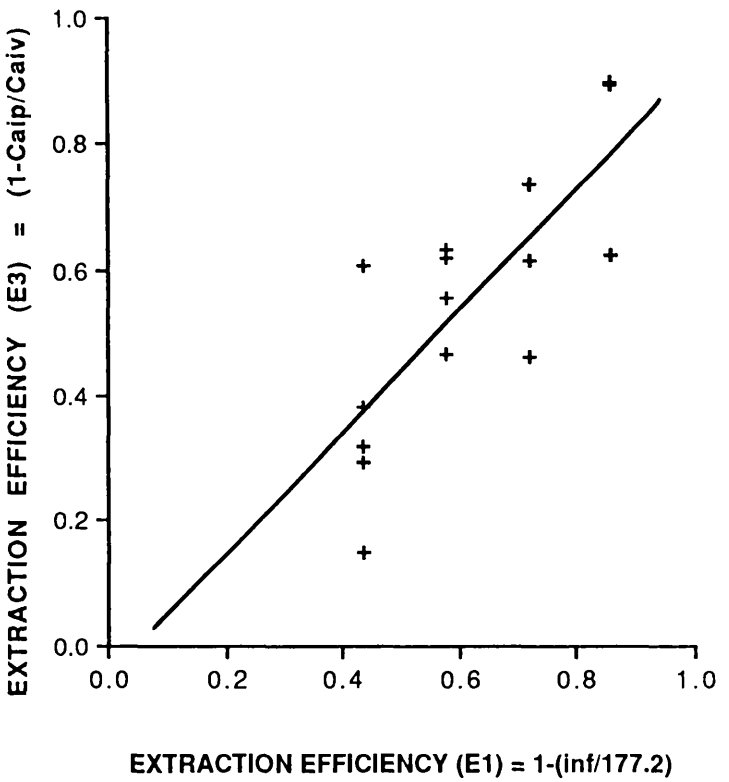


Figure 27

The relationship between the extraction efficiency expression $1-(Ca^{ip}/Ca^{iv})$, derived from the intraportal infusion of galactose in sham operated rats and denoted by E_3, and the extraction efficiency expression 1-(inf/177.2) denoted by E_1 is described by the linear regression equation $E_3 = 1.0E_1 - 0.0657$. A correlation coefficient of 0.784 was found.

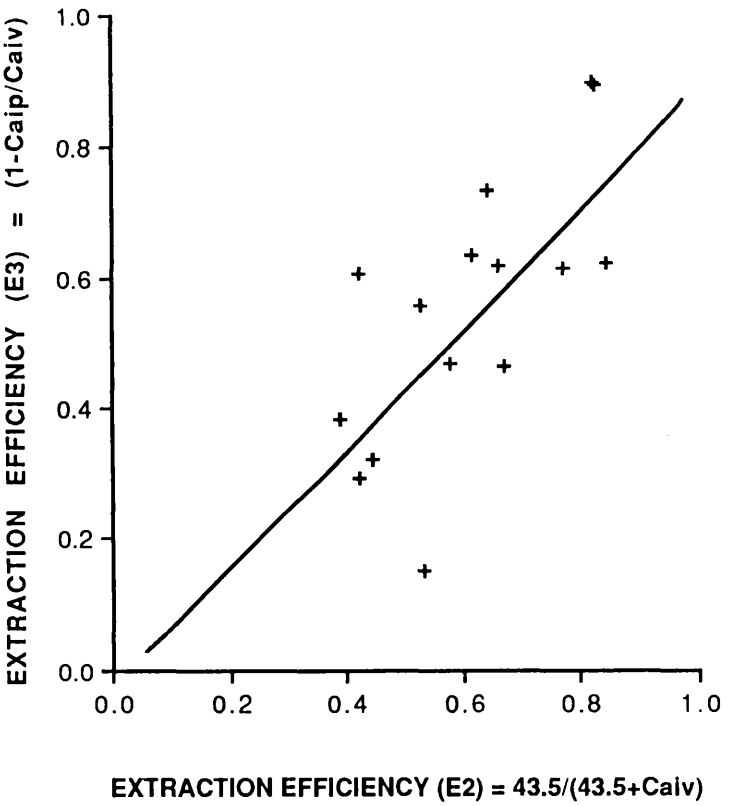


Figure 28

This shows the relationship between the extraction efficiency expression $1-(Ca^{ip}/Ca^{iv})$ denoted by E_3 and the extraction efficiency expression $43.5/(43.5+Ca^{iv})$ denoted by E_2. The relationship is described by the linear regression equation $E_3 = 0.96E_2 - 0.036$. A correlation coefficient of 0.712 was found.

TABLE 18

Infusion Rate (mg/kg/hr)	No of animals	Expressions of Extraction Efficiency		
		E1	E2	E3
		$[1-(V/Vmax)].Eo$	$Eo.Km/(Km+Cm)$	$1-Ca^{ip}/Ca^{iv}$
		Mean	Mean ± SEM	Mean ± SEM
25	3	0.859	0.830 ± 0.008	0.804 ± 0.09
50	3	0.718	0.694 ± 0.039	0.606 ± 0.078
75	4	0.577	0.594 ± 0.028	0.571 ± 0.038
100	5	0.436	0.442 ± 0.024	0.351 ± 0.075
125	3	0.295	0.304 ± 0.015	---------

This shows the mean ± SEM hepatic extraction efficiency values for all three extraction efficiency expressions at different infusion rates in sham operated rats. There was no statistically significant difference between E_1, E_2 and E_3 at any infusion rate.

INTRAVENOUS INFUSION OF GALACTOSE IN PORTAL VEIN LIGATED RATS

Relationship between equilibrium blood galactose concentration and infusion rate

Returning to the Michaelis-Menten equation,

$$V = Vmax.Ca^{iv}/(Km + Ca^{iv}) \quad \ldots\ldots\ldots\ldots\ldots\ldots 7.1$$

Again the parameters Vmax and Km in portal vein ligated rats can be estimated from experimental studies carried out in which equilibrium blood galactose concentrations at various intravenous infusion rates were measured. Equilibrium blood galactose concentrations were achieved as previously shown in sham operated rats (Figure 23). Figure 29 shows the relationship between infusion rates and equilibrum concentrations, and by non-linear regression analysis (Statgraphics, statistical graphics system) of the hyperbola, the parameters Vmax and Km were estimated to be 180.09 $\pm$ 27.44 mg/kg/hr and 56.42 $\pm$ 17.48 mg/l (mean $\pm$ SEM) respectively. The mean values of Vmax and Km are not apparently changed from the values estimated from the intravenous infusion in sham operated rats, however, there was more scatter in data, and thus presumably more variability in individual values of Vmax and/or Km.

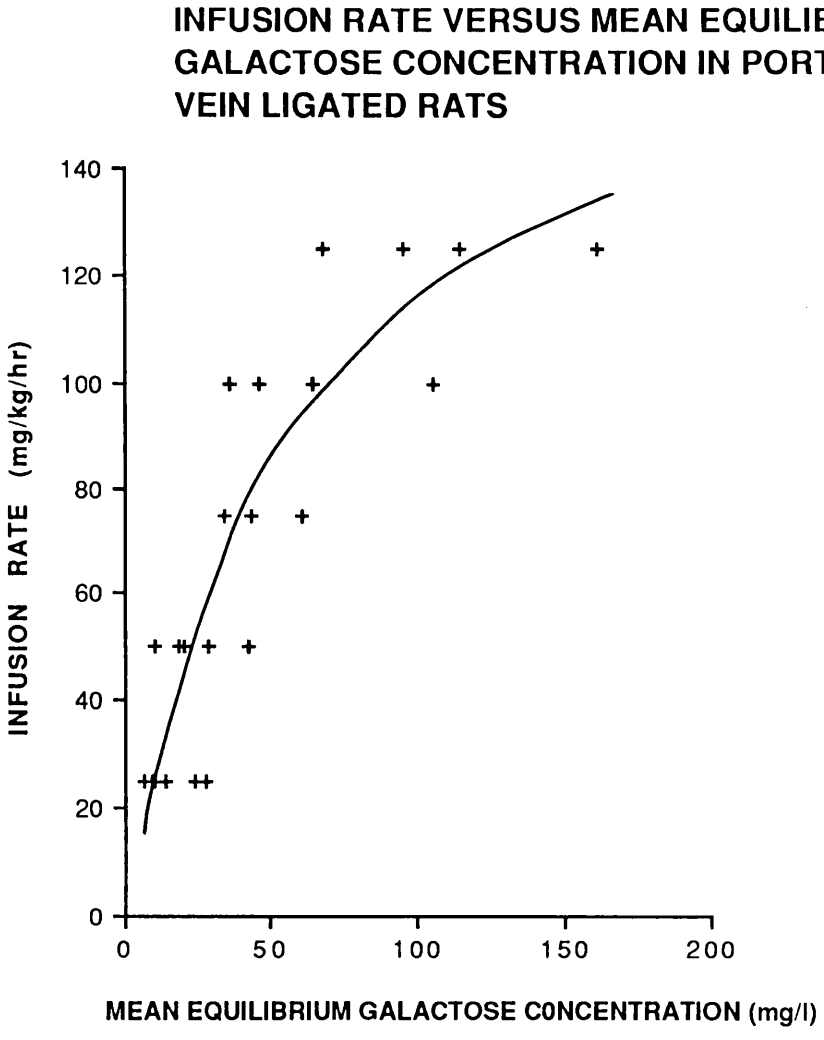


Figure 29

This shows the relationship between mean equilibrium blood galactose concentration at various intravenous infusion rates in portal vein ligated rats. The parameters Vmax and Km of the curve were found, by non-linear regression analysis, to be 180.09 ± 27.44 mg/kg/hr and 56.42 ± 17.48 mg/l (Mean ± SE) respectively.

INTRAPORTAL INFUSION OF GALACTOSE IN PORTAL VEIN LIGATED RATS

Comparison of systemic availability of intraportal galactose in portal vein ligated and sham operated rats

Since portal vein ligated animals undergo portasystemic shunting, hepatic portal flow (Fhp) is equal to flow in the portal vein (Fpv) multiplied by (1 - S), where S represents portasystemic shunting expressed as a fraction, ie

$$Fhp = Fpv(1 - S) \quad \text{.....7.24}$$

If we return to equation 7.20:

$$I = E(F_1 Ca^{ip} + IFhp/Fpv) \quad \text{.....7.20}$$

then by inserting equation 7.24 into equation 7.20,

$$I = E(F_1 Ca^{ip} + I(1 - S)) \quad \text{.....7.25}$$

by rearranging,

$$Ca^{ip} = I(1 - E + ES)/EF_1$$

Since $I/E.F_1 = Ca^{iv}$ (equation 7.22), then

$$Ca^{ip} = Ca^{iv}(1 - E + ES) \quad \text{.....7.26}$$

$$Ca^{ip}/Ca^{iv} = 1 - E + ES$$

$$= 1 - E(1 - S) \quad \text{.....7.27}$$

$$\text{or} = S + (1 - S)(1 - E) \quad \text{.....7.28}$$

Ca^{ip}/Ca^{iv} = Systemic availability (SA) as defined in Chapter 2.

When S = 0, equation 7.28 becomes equivalent to equation 7.23 as derived for sham operated rats.

Table 19 shows the mean systemic availability values calculated from the expression Ca^{ip}/Ca^{iv} at four different infusion rates in both sham operated and portal vein ligated rats. Systemic availability does not vary significantly with infusion in portal vein ligated rats: by ANOVA a p value of 0.373 was found. However, in sham operated rats systemic availability does vary significantly with infusion, a p value of 0.0071 was found by ANOVA. The increasing systemic availability in sham operated rats reflects the increasing saturation of the galactose extraction mechanism with concentration. The more constant systemic availability in portal vein ligated rats reflects the fact that a constant fraction of galactose is diverted via shunts irrespective of concentration and hepatic extraction efficiency. Figure 30 shows mean systemic availability (Ca^{ip}/Ca^{iv}) values in sham operated and portal vein ligated rats. As infusion rates increase systemic availability in sham operated rats converges towards the systemic availability in portal vein ligated rats. Error bars clearly indicate the SEM at each mean value.

TABLE 19

Infusion Rates (mg/kg/hr)	Systemic availability	
	PVL	SO
	Mean ± SEM	
25	0.648 ± 0.090	0.196 ± 0.090
50	0.762 ± 0.076	0.394 ± 0.078
75	0.763 ± 0.064	0.429 ± 0.038
100	0.831 ± 0.038	0.649 ± 0.075
Overall Mean ± SEM	0.750 ± 0.037	0.449 ± 0.036

PVL = Portal vein ligated
SO = Sham operated

This shows the mean ± SEM values for Ca^{ip}/Ca^{iv} at four different infusion rates in portal vein ligated and sham operated rats. Systemic availability did not vary significantly with infusion in portal vein ligated rats ($p=0.373$ by ANOVA). In sham operated rats a p value of 0.0071 was found (ANOVA) and systemic availability varied significantly with infusion rate.

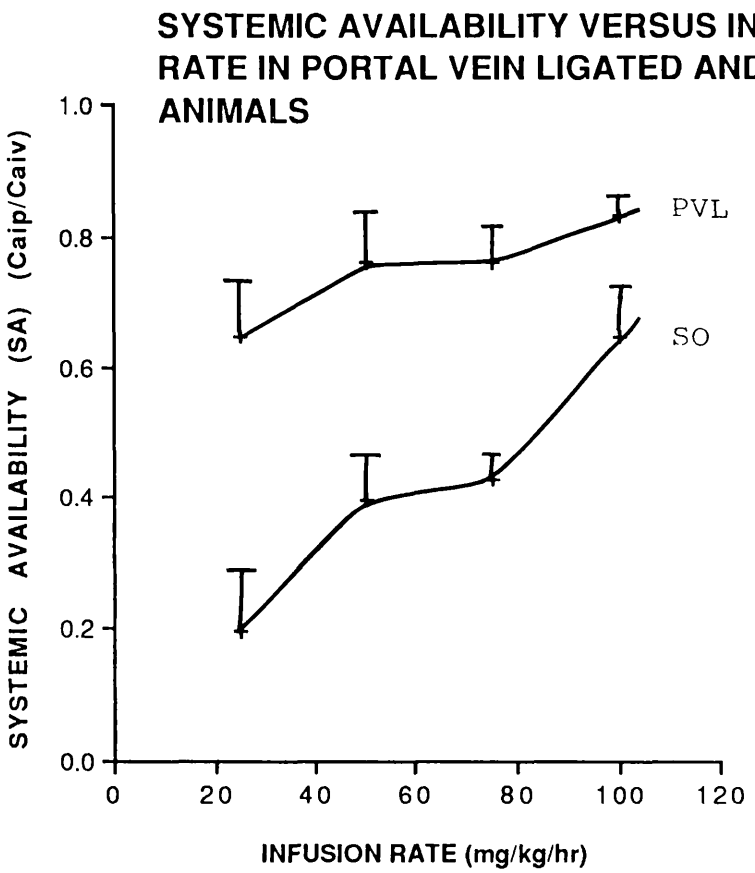


Figure 30

This shows the systemic availability (Ca^{ip}/Ca^{iv}) of galactose at four different infusion rates in portal vein ligated (PVL) and sham operated rats (SO). In sham operated rats systemic availability varied significantly with infusion rate ($p = 0.007$). Error bars indicate the SEM at each mean value.

Relationship between portasystemic shunting calculated from microspheres and infusion rate

Returning to equation 7.28 and rearranging,

$$S = [(Ca^{ip}/Ca^{iv}) - (1 - E)]/E \quad \text{.....7.29}$$

If Eo is assumed to be 1, then by inserting equation 7.14, $E = [1 - (V/Vmax)].Eo$, into equation 7.29, an expression for shunting is derived,

$$S_1 = [(Ca^{ip}/Ca^{iv}) - (I/Vmax)]/(1 - I/Vmax) \quad \text{....7.30}$$

A second expression for shunting is derived if equation 7.12, $E = Eo.Km/(Km + Ca^{iv})$, is inserted into equation 7.29, and Eo is assumed to be 1,

$$\begin{aligned} S_2 &= (Ca^{ip}/Ca^{iv} - [Ca^{iv}/(Km + Ca^{iv})(Km + Ca^{iv}/Km) \\ &= Ca^{ip}/Ca^{iv} + Ca^{ip}/Km - Ca^{iv}/Km \\ &= Ca^{ip}/Ca^{iv} - (Ca^{iv} - Ca^{ip})/Km \quad \text{.....7.31} \end{aligned}$$

In all experiments portasystemic shunting was also calculated from the injection of microspheres into the portal vein as described in chapter 5. The mean $\pm$ SEM values of portasystemic shunting calculated from microsphere injection and from the two expressions for portasystemic shunting in both sham operated and portal vein ligated rats are shown in Table 20. From the table it can be seen that there is a tendency, which is

non-significant, for the shunting expressions to overestimate portasystemic shunting in sham operated rats, and to significantly underestimate, $p<0.01$, portasystemic shunting in portal vein ligated rats.

The relationships between portasystemic shunting determined from microspheres and that calculated from both of the above expressions of shunting in both sham operated and portal vein ligated rats are described in Figures 31 and 32, where the mean values of Km and Vmax respectively have been used.

The relationship between portasystemic shunting (PSS), as measured by microspheres and equation $S_1 = [(Ca^{ip}/Ca^{iv} - I/Vmax)]/(1 - I/Vmax)$ is described by the linear regression equation $S_1 = 13.75 + 0.55PSS$. A correlation coefficient of 0.706 was found (Figure 31). The standard error (SEM) of the intercept and slope were 6.2 and 0.097 respectively. Figure 32 shows the relationship between portasystemic shunting (PSS), as measured by microspheres and equation $S_2 = Ca^{ip}/Ca^{iv} - (Ca^{iv} - Ca^{ip})/Km$. The relationship is described by the equation $S_2 = 11.94 + 0.49PSS$. A correlation coefficient of 0.605 was found. The standard error (SEM) of the intercept and slope were 7.32 and 0.11 respectively.

Individual variations in Km and Vmax values presumably account for some of the discrepancies between portasystemic shunting, as measured by

microspheres and that calculated from shunting equations S_1 and S_2. However, as shown in Table 20 there was also a systematic tendency for galactose shunting values in portal vein ligated rats to be lower than the microsphere values. This finding will be discussed more fully in the following discussion.

TABLE 20

Procedure	Portasystemic Shunting (microspheres) (%) Mean ± SEM	Expressions for Portasystemic Shunting S_1 (%) Mean ± SEM	S_2 (%) Mean ± SEM
SO	1.0 ± 0.00	11.0 ± 6.0	10.0 ± 7.0
PVL	83.0 ± 5.0	62.0 ± 5.0 *	55.0 ± 7.0 *

SO = Sham operated rats
PVL = Portal vein ligated rats

* $p < 0.01$ galactose versus microsphere measurements (paired t-test)

This shows the mean ± SEM portasystemic shunting values calculated from injection of microspheres into the portal vein, and from the shunting expressions S_1 and S_2 derived from the kinetic model. Galactose shunting values (S_1 and S_2) in portal vein ligated rats were significantly lower than the microsphere values.

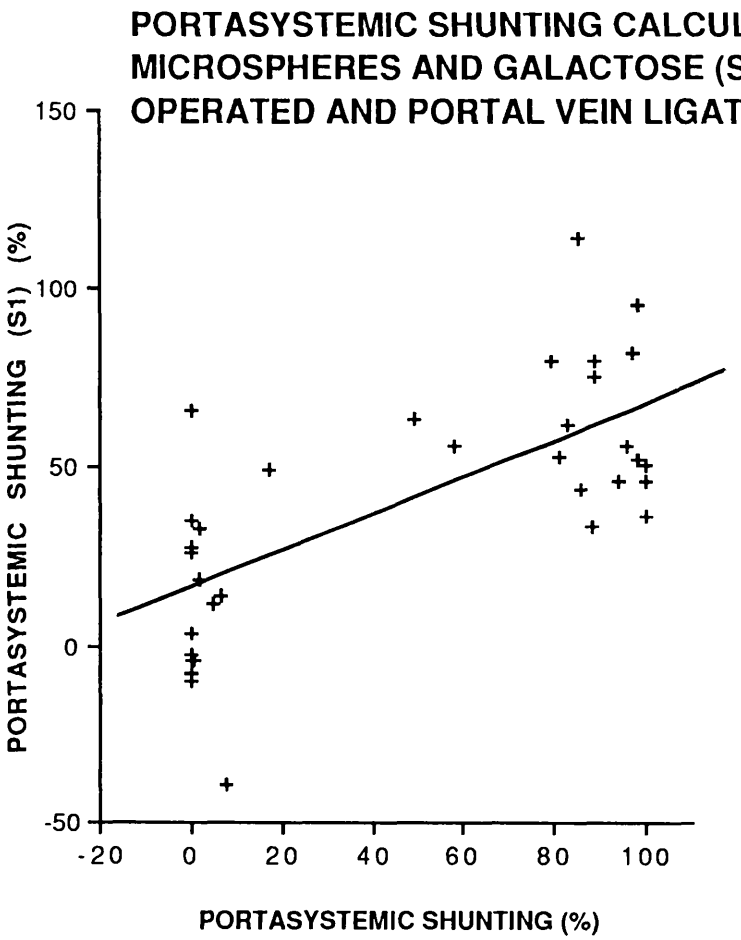


Figure 31

This shows the relationship between portasystemic shunting calculated from the equation $S = (Ca^{ip}/Ca^{iv} - I/Vmax)/(1 - I/Vmax)$ and portasystemic shunting (PSS), as measured by microspheres, in both sham operated and portal vein ligated rats. The relationship is described by the linear regression equation $S_1 = 13.75 + 0.55PSS$. A correlation coefficient of 0.706 was found.

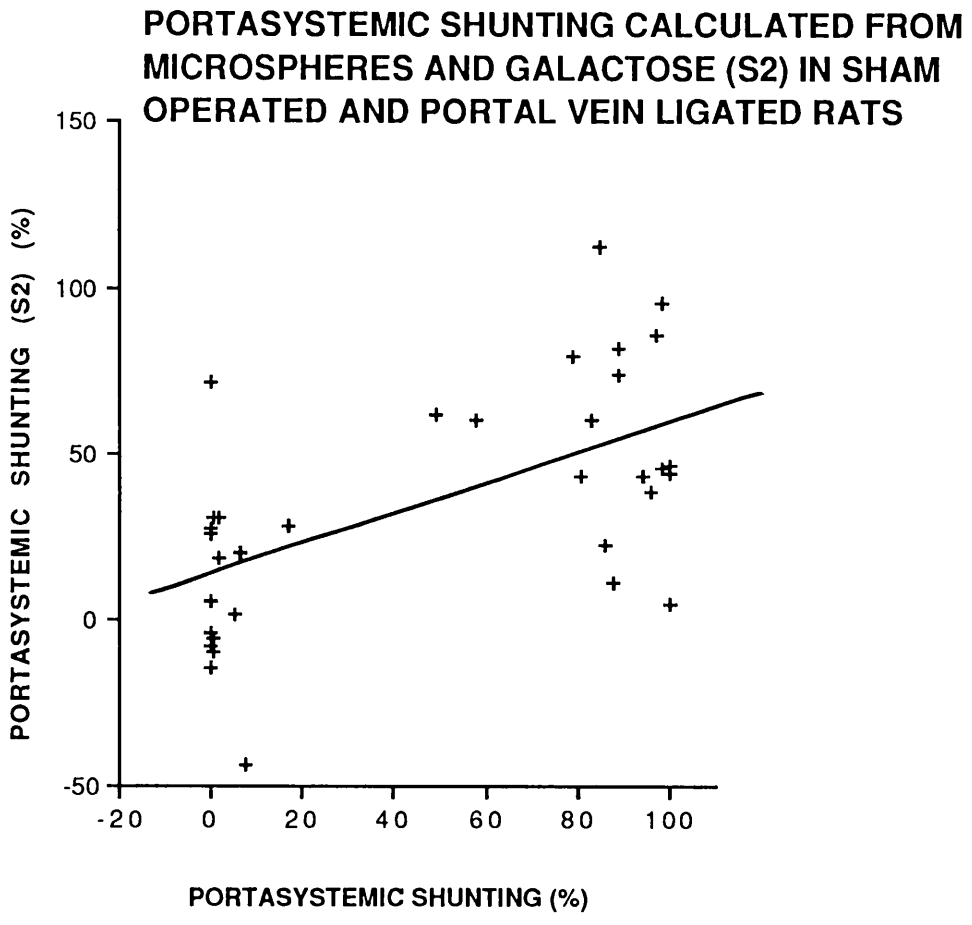


Figure 32

This shows the relationship between portasystemic shunting calculated from the equation $S_2 = Ca^{ip}/Ca^{iv} - (Ca^{iv} - Ca^{ip})/Km$ and portasystemic shunting (PSS), as measured by microspheres, in portal vein ligated and control animals. The relationship is described by the linear regression equation $S_2 = 11.94 + 0.49PSS$. A correlation coefficient of 0.605 was found.

GALACTOSE CLEARANCE AT DIFFERENT INTRAVENOUS INFUSION RATES IN SHAM OPERATED AND PORTAL VEIN LIGATED RATS

By definition, hepatic clearance (Cl) is the volume of blood from which galactose is completely removed by the liver in unit time (Chapter 2). This is given by the elimination rate of galactose by the liver (V) divided by the mean concentation of galactose in blood (Cm),

ie. $V/Cm = Cl$ 7.32

Clearance would translate into liver blood flow (F_l) if it was assumed that there was complete extraction of galactose from each ml of blood flowing through the liver.
Comparing equation 7.32 with equation 7.7 ($E=V/F_lCm$), it can be seen that clearance equals EF_l.

ie. $Cl = EF_l$ 7.33

In these experiments we have measured systemic clearance of galactose, given by I/Ca^{iv}, which we equate with hepatic clearance under Assumption 2 (V=I). Equation 7.33 can thus be rewritten,

$$I/Ca^{iv} = E.F_l \quad \text{.....7.34}$$

The mean clearance values (I/Ca^{iv}) of galactose at five different infusion rates in sham operated and portal

vein ligated rats are shown in Table 21. Mean hepatic clearance values in both groups at each of the five infusion rates are not significantly different from each other (2-way ANOVA). In both groups clearance decreased as infusion rate increased ($p<0.05$), reflecting the decline in hepatic extraction efficiency.

When the mean systemic clearance values found in this chapter were compared to those found from the intraportal infusion of galactose in chapter 6, systemic clearance of galactose by both routes proved to be very similar. The mean systemic clearance value calculated in chapter 6 was derived from the slope of the galactose clearance curve and was dependent upon the volume of distribution (Vd). The volume of distribution found in a study by Keiding (1973) was 41 percent of body weight. If this value is taken to be a reliable estimate, then the mean clearance values in min^{-1} of chapter 6 translated into l/hr in sham operated and portal vein ligated rats were 0.59 and 0.58 l/hr respectively. The equivalent translated mean clearance values in this chapter were 0.64 and 0.58 l/hr in sham operated and portal vein ligated rats respectively. The similarity in clearance values calculated from the two different routes of infusion demonstrates the consistency of the model and the soundness of the methods applied.

TABLE 21

Infusion Rate (mg/kg/hr)	Clearance (l/kg/hr) PVL	SO
	Mean ± SEM	
25	2.156 ± 0.444	2.808 ± 0.161
50	2.560 ± 0.498	2.752 ± 0.552
75	1.726 ± 0.273	2.582 ± 0.300
100	1.922 ± 0.311	1.854 ± 0.194
125	1.256 ± 0.227	1.257 ± 0.092
Overall Mean ± SEM	2.005 ± 0.190	2.225 ± 0.129

PVL = Portal vein ligated
SO = Sham operated

This shows the mean ± SEM clearance (I/Ca^{iv}) values at five different intravenous infusion rates in sham operated and portal vein ligated rats. In both groups clearance decreases as infusion rate increases ($p<0.05$), but there was no significant difference in clearance between portal vein ligated and sham operated rats at any infusion rate (2-way ANOVA).

EXTRAHEPATIC ROUTE FOR GALACTOSE ELIMINATION

One of the fundamental assumptions underlying the kinetic theory described in this chapter has been the assumption that the liver is the major organ of galactose elimination. This assumption can be examined more fully by quantifying possible extrahepatic pathways for galactose elimination. Possible routes for extrahepatic elimination of galactose are urinary excretion and/or renal metabolism and erythrocyte metabolism. Keiding (1985) has shown that galactose metabolism in the erythrocytes is of no quantitative importance. Urinary excretion, however, proved more significant as an extrahepatic pathway for galactose removal, but was still quantitatively small, an average value of 9 percent of the amount galactose given was found by Keiding (1973).

In order to eliminated the possibility that urinary excretion may be quantitatively important in the context of this study and substantiate to some extent the assumption that the liver is the major organ of galactose elimination, the percent galactose appearing in urine was calculated after a two hour continuous infusion of galactose at rates of 75 and 125 mg/kg/hr. Table 22 shows the mean $\pm$ SEM percent urinary excretion of galactose in sham operated and portal vein ligated rats. At an infusion rate of 75 mg/kg/hr percent

urinary excretion was 1.57 ± 0.29 and 0.81 ± 0.46 (mean ± SEM) in portal vein ligated and control rats respectively. A mean ± SEM percent urinary excretion of 5.14 ± 0.29 and 3.35 ± 0.05 was found at an infusion rate of 125 mg/kg/hr in both portal vein ligated and sham operated rats respectively. The values found in this study are quantitatively small, implying that extrahepatic elimination of galactose by the kidneys is negligible. The possible existence of alternative, as yet unidentified pathways for galactose elimination cannot be ruled out. However, at present it would appear that the original assumption that the liver is the major organ of galactose elimination is correct.

TABLE 22

Procedure	Infusion rates (mg/kg/hr)	
	75	125
	Urinary excretion (%)	
	Mean ± SEM	
SO	0.81 ± 0.46 (n=4)	3.35 ± 0.05 (n=2)
PVL	1.57 ± 0.29 (n=5)	5.14 ± 0.29 (n=5)

PVL = Portal vein ligated
SO = Sham operated

This shows mean ± SEM percent urinary excretion in sham operated and portal vein ligated rats at two different infusion rates.

DISCUSSION

The purpose of the study carried out in this chapter was to examine whether or not poor extraction efficiency in sham operated rats could explain the failure to see any differences in systemic availability of galactose following oral and intraportal doses of galactose, as described in the previous chapter.

It has been shown that hepatic uptake of galactose by the hepatocyte follows Michaelis-Menten kinetics (Keiding et al, 1976; Keiding and Chiarantini, 1978). It has also been shown by Keiding and Bass (1983), that from the Michaelis-Menten equation, $V = VmaxCm/(Km+Cm)$, a formula for extraction efficiency (E) can be developed, where $E = 1-\exp[1-(Vmax-I)]/F.Km$. The symbols I and F represent galactose infusion rate and hepatic liver blood flow respectively. In this equation, extraction efficiency of the hepatocyte is dependent upon the parameters Vmax, Km, and galactose infusion rate, all of which can be readily determined, and the unknown, and not readily determined parameter hepatic blood flow. This unknown parameter makes the solution of the equation rather complicated. An alternative and simpler approach in the intact rat is to apply the Michaelis-Menten equation to the liver as a whole. Thus V, and Vmax would represent the galactose elimination rate and its maximum value for the liver as

a whole, and Km the concentration of galactose in the systemic circulation at which elimination is half maximal. By applying Michaelis-Menten type kinetics to the whole liver expressions of hepatic extraction efficiency can be derived which are more readily solved. Keiding (1973) has shown this approach to produce Km and Vmax values in reasonable agreement to those found for the hepatocyte (Keiding et al, 1976). However, the Km value for galactose in blood is higher, 72 mg/l (Keiding, 1973) than 'Km' calculated on the basis of galactose concentration in liver tissue, between 21.6 and 54 mg/l (Keiding et al, 1976), owing to the decreasing galactose concentration as the blood flows through the liver (Keiding, 1973).

In this study Michaelis-Menten kinetics were applied in the intact rat and the self-consistency of the kinetic model examined by both intravenous and intraportal infusions in sham operated rats.

The continuous-infusion technique used in this study was chosen for the following reasons. First, it reduces distribution phenomena of galactose and other metabolites in the circulatory system as well as the intracellular spaces of the liver. Simultaneous steady-state estimates of the galactose elimination rate and blood concentrations were ensured, and this facilitates the comparison of estimates.

The mathematical kinetic model (equation 7.1) assumes a

time-independent concentration of galactose, i.e., dc/dt = 0. In all experiments in both the intraportal and intravenous infusions of galactose this equilibrium state was achieved. For these reasons the steady-state experiments are assumed to provide reliable estimates of the kinetic parameters.

The model describes the relation between the galactose elimination rate, V, (in this study V equals galactose infusion rate, I, at equilibrium), and the mean equilibrium systemic blood galactose concentration, Cm, by the Michaelis-Menten equation. The model was tested by plotting systemic blood galactose concentrations against increasing intravenous infusions of galactose in sham operated rats. According to the model, the rate should asymptotically approach a maximum, and saturation kinetics should be followed. Results from the experiments were consistent with Michaelis-Menten type kinetics and the hepatic elimination of galactose was described kinetically by the parameters Vmax and Km of the model. The mean estimates of Km and Vmax in this study were determined with relatively small errors but the systemic availability of galactose at a given infusion rate showed significant individual differences. The interindividual variation includes probably both individual differences between the enzymatic reactions in different livers for galactose elimination and variations between experimental factors

which will be discussed later. It should also be pointed out that the parametrization of the model by Vmax and Km implies that the estimates of the parameters become highly correlated, and therefore caution should be exercised when the estimates are used for testing various hypotheses about the parameters.

The parameters Vmax and Km are themselves thought to describe one of the enzymatic steps in the metabolic conversion of galactose. The first step in the main pathway of the hepatic conversion of galactose involves phosphorylation to galactose-1-phosphate with consumption of ATP by the enzyme galactokinase (EC.2.7.1.6). It is this practically irreversible galactokinase step which is considered to be rate limiting in the elimination of galactose by the liver and which the parameters Vmax and Km describe (Cuatrecausas and Segal, 1965; Keiding, 1973).

On comparison of the Vmax and Km values found in this study, 177.2 mg/kg/hr and 43.5 mg/l repectively, and those from studies carried out by Keiding (1973), and Salaspuro and Salaspuro (1968), it was found that our Km value was smaller and Vmax value greater. Keiding (1973) demostrated a Km value of 72 mg/l and both Salaspuro and Salaspuro (1968), and Keiding (1973) found Vmax values of 13.2 and 12.04 mg/100g/hr repectively. Our equivalent translated Vmax value was 17.72 mg/100g/hr. The Km value is of particular

importance since it gives us an insight into the dependency of hepatic extraction efficiency on circulating blood galactose concentrations. In this study the level of Km was such that the extraction efficiency term Km/(Km + Cm) was highly concentration dependent in the range 0 to 100 mg/l. Such high dependency on concentration in this range was not entirely expected since previous studies had reported Km values of 72 mg/l (Keiding, 1973). This level of dependency may in part explain why the oral/intraportal doses showed relatively high systemic availability in sham operated rats. In this chapter we look at equilibrium blood galactose concentrations. It should be borne in mind that an oral dose may give rise to high transient concentrations in the portal vein, hence giving low extraction efficiency. The results also suggest high variability in individual Km and Vmax values, which would further tend to mask differences between groups with respect to the systemic availability of oral and intraportal doses when the liver is near saturation.

In this kinetic model it would seem that galactose elimination kinetics are independent of liver blood flow (F_l). The apparent absence of explicit dependence on liver blood flow is misleading, as galactose elimination kinetics depends on hepatic blood flow through equation 7.10, $Vmax/Km.F_l = Eo$, where Eo is the

hepatic extraction efficiency of galactose at low systemic galactose concentrations. This dependence of galactose elimination kinetics on hepatic blood flow is described through the sinusoidal perfusion theory (Keiding and Chiarantini, 1978). If blood flow varies, for example through a change in sinusoidal blood velocity without affecting the number of sinusoids perfused this is unlikely to affect Vmax and Km will vary inversely with flow. Therefore an increasing flow rate throught the liver will reduce hepatic transit time and so consequently reduce Km, thus systemic blood galactose concentration will increase as less galactose is extrated by the liver. The reverse will occur if liver blood flow is reduced. Alternatively, if flow varies through a change in the number of perfused sinusoids without changing blood velocity it will be Vmax rather than Km that changes.

A possible explanation of differences in Km and Vmax values in our study to those found by Keiding (1973) and Salaspuro and Salaspuro (1968) can perhaps be explained by the effect of anaesthesia and the type of anaesthesic used, the type of surgery applied, and animals differences, such as animal strain and sex on hepatic blood flow. Salaspuro and Salaspuro (1968) found that the elimination of galactose was temporarily blocked by penthotal anaesthesia, and thus subsequently in all experiments used unanesthetised rats. Keiding

(1973), however applying the same anaesthetic did not find galactose elimination to be affected. A systematic influence of anaesthesia on the results obtained, however cannot be excluded. Further, it was assumed in our study that surgical trauma, such as cannulation of the portal vein was of minor importance. The self-consistency of the theory was tested by comparing the extraction efficieny term Km/(Km+Cm), with alternative expressions of extraction efficiency derived from the intraportal and intravenous infusions of galactose, whose solution did not depend on the parameter Km. The relationship found between these expressions were linear and close to the line of identity. This finding thus reinforces the validity of the theory and our approach to applying Michaelis-Menten kinetics to the whole organ.

From the intraportal infusion of galactose two expressions for shunting were derived (equations 7.30 and 7.31). Using the mean values of Vmax and Km both equations can be solved to give portasystemic shunting values for each animal individually, which in turn can be compared with the independent assessment of portasystemic shunting from injected microspheres. In sham operated rats all three methods compared favourably. However, in portal vein ligated rats there was a tendency towards the shunting expressions derived from the kinetic model to underestimate the degree of

portasystemic shunting as compared to that measured by injected microspheres. The reason for this is unclear but it may well be that injected microspheres are not as a reliable indicator of the magnitude of portasystemic shunting as previously supposed. Microspheres enter portal venous blood in the physical form of radioactive particles whereas galactose enters the circulation as a solution. This difference in the physical state between galactose and microspheres may in part contribute to the observed differences in their assessment of the magnitude of portasystemic shuning. Further, a particular source of measurement error which is commonly encountered with radioactive microspheres is that arising from inadequate mixing of injected microspheres (Buckberg et al, 1971). If this occurs, streamlining of microspheres with blood will prevail and produce inaccurate assessment of the magnitude of portasystemic shunting. Furthermore, if indeed portal vein ligated and sham operated rats are not so different in the magnitude of portasystemic shunting as suggested by the galactose expressions for portasystemic shunting, then the systemic availability of galactose would in itself be less differentiated between control and portal hypertensive rats following an oral dose of galactose.

One of the main assumptions in this chapter has been that the liver is the major organ of galactose

extraction. This appears to be a reasonable assumption since many investigators have shown this to be the case (Roe and Cowgill, 1935; Goresky et al, 1973). Further, alternative routes of galactose elimination, such as urinary excretion, renal metabolism and erythrocyte metabolism have been shown to be negligible (Keiding, 1973; McNamara and Segal, 1972; Keiding, 1985). In this study we examined urinary excretion of galactose, which proved to be very low at the concentrations attained. However, we cannot rule out the possibility that other metabolic pathways for galactose elimination may exist, but the good approximation between extraction efficiency as calculated from Ca^{ip}/Ca^{iv} and extraction efficiency as calculated from the characteristics of the intravenous infusion rate versus concentration curve suggests that such extrahepatic extraction is small. This is because the former is based on a comparison of intraportal and intravenous infusions which will clearly depend on hepatic extraction (irrespective of other pathways), whereas the latter is dependent on systemic clearance which is influenced by both hepatic and extrahepatic elimination rates.

CONCLUSIONS

Oral and intravenous administration of galactose is a non-invasive, physiological method of measuring the magnitude of portasystemic shunting occuring in portal hypertension. The technique applied was a considerable improvement on earlier methods for assessing the magnitude of portasystemic shunting, avoiding invasive and time consuming measurements and overcoming measurement inaccuracies. The technique was easily comprehensible, readily applicable, safe and well accepted by the patients.

The galactose dehydrogenase enzyme assay coupled with fluorescence spectrophotometry was well able to measure the systemic galactose concentrations encountered in this study. The enzyme assay was simple, accurate and easy to use.

The assumptions underlying the galactose measurement technique were tested independently in control volunteers from the University Department of Surgery, Glasgow Royal Infirmary. The prerequisite of adherance of galactose elimination to first-order kinetics at the intravenous infusion rates used in this study was followed in all control volunteers studied. The oral dose of galactose chosen was consistent with high extraction efficiency in the normal liver and thus effectively complete clearance from blood on first pass

through the normal liver following absorption from the small intestine.

Data from the patient study carried out in this thesis showed there was a significant difference in the systemic availability of galactose between control patients and patients with portal hypertension and/or liver disease ($p < 0.005$). No independent method for quantitation of the magnitude of portasystemic shunting was performed on patients in this study as alternative methods required surgical access to the splanchnic blood vessels (Groszmann et al, 1972) and it was not considered ethically justified to perform such vaildatitory measurements on the patients. However, portasystemic shunting values from this study are in good agreement with those found in other studies (Groszmann et al, 1972; Okuda et al, 1977).

The application of the technique in the prehepatic rat model of portal hypertension allowed the measurement of the magnitude of portaystemic shunting to be assessed objectively, in that independent measurements of portasystemic shunting could be performed using a radioactive microsphere technique.

The initial failure to show a difference in the systemic availability of galactose between control and portal hypertensive rats was attributed in the main to the erratic and incomplete absorption of galactose from the gastrointestinal tract. The retention of

galactose in the gastrointestinal tract does not appear to occur in man, as evidenced by the rapid rise and peak of systemic galactose concentrations approximately 10 to 20 minutes following oral administration of galactose and the finding that a systemic availability of galactose of close to 100 percent was achieved in several portal hypertensive patients. The possibility of retention in a minority of patients however, cannot be excluded but it is difficult to test definitively. The accuracy of the technique in this animal model, in contrast to man, was further compromised when a more comprehensive study into the kinetics of hepatic galactose elimination in the rat model, by direct infusion of galactose into the portal vein, showed the hepatic extraction efficiency for galactose to be highly dependent on circulating blood galactose concentrations. Hepatic galactose extraction can be described by Michaelis-Menten kinetics in which the kinetic parameters Vmax and Km repesent the maximum galactose elimination rate and the galactose concentration at which hepatic galactose elimination is half maximal respectively. In this study the kinetic parameter Km, was found to have a value of 43.5 mg/l. By contrast in human volunteers, systemic galactose clearance was found to be independent of concentration up to levels in the region of 42 mg/l. The variation in the values of the kinetic parameters between animals

make quantitation of the magnitude of portasystemic shunting by galactose in rats inaccurate at the individual level, but despite this finding a highly significant correlation between the systemic availability of intraportally administered galactose in rats and portasystemic shunting, as measured from the injection of radiolabelled microspheres was found.

It is unclear why there is a difference between rats and humans with respect to both absorption of oral galactose from the gastrointestinal tract and hepatic elimination of galactose. It is possible that the difference is a result of species difference and the fact that all animals were anaesthetised throughout the galactose experiments. Despite this somewhat disappointing contrast, the results obtained in patients with liver disease and/or portal hypertension are compatible with expectations.

To date there is no fixed quantitative definition of portasystemic shunting. Indeed quantitation of anatomical shunting varies according to the vascular bed studied (Groszmann et al, 1972). The fact that both functional and anatomical shunting have been examined in this study may further compound the problem. Therefore, whether the systemic availability of galactose should be regarded as a measure of portasystemic shunting is largely a matter of definition.

The potential clinical usefulness of the method described in this thesis is likely to rest on the possibility that other gut-derived agents such as hormones, nutrients and drugs behave as, and share their fate with galactose. An increasing systemic availability of galactose should therefore parallel the appearance and progression of clinical problems associated with portal hypertension. The most likely area for future studies would be in hepatic encephalopathy, the aetiology of which has been associated with the effects of circulatory changes in portal hypertension (Phear et al, 1955). This line of future investigation, however, can only be explored experimentally.

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