PORTACAVAL TRANSPOSITION IN THE RAT

A new model for investigation of portal-systemic encephalopathy

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КЪ ВОПРОСУ О ПЕРЕВЯЗКѢ ВОРОТНОЙ ВЕНЫ.

Предварительное сообщеніе.

Д-ра Н. Экка.

Если собакѣ перевязать воротную вену, предварительно обезпечивъ крови этой вены свободный оттокъ въ нижнюю полую вену, то эта перемѣна въ направленіи кровянаго тока и лишеніе печени крови воротной вены никакихъ тяжкихъ разстройствъ въ организмѣ не производятъ; животное отъ операціи выздоравливаетъ, питаніе его по мѣрѣ выздоровленія поправляется и остается далѣе въ отличномъ состояніи.

Путемъ многихъ опытовъ, въ томъ числѣ болѣе 60 вивисекцій, я выработалъ удобный способъ образованія свища между сказанными венами. Я сшиваю тонкимъ шелкомъ тремя продольными швами лѣвый бокъ передней поверхности нижней полой вены съ лѣвымъ бокомъ задней поверхности воротной вены; затѣмъ такимъ-же рядомъ симметричныхъ съ первыми швовъ— правый бокъ задней поверхности воротной вены съ передне-правой частью поверхности нижней полой вены.

Вены, такимъ образомъ сшитыя, соприкасаются приблизительно равными участками. На этихъ площадяхъ, ограниченныхъ обоими рядами швовъ, продольнымъ разрѣзомъ стѣнокъ образуется между сшитыми венами свищъ. Внутрисосудистое давленіе прижимаетъ края разрѣзовъ одной вены къ краямъ разрѣзовъ другой настолько, что задерживаетъ всякое кровотеченіе наружу.

Для разрѣзыванія венъ я устроилъ небольшія изогнутыя по плоскости (подъ угломъ) ножницы, къ концамъ которыхъ припаяно по тонкой длинной серебренной проволокѣ, а къ концамъ проволокъ по стальной иглѣ.

Послѣ того, какъ первымъ рядомъ швовъ лѣвыя стороны венъ сшиты, и до наложенія втораго ряда швовъ опредѣляется примѣрно на глазъ мѣсто предполагаемаго разрѣза на каждой изъ венъ; на всю длину этого разрѣза въ ту и другую вены вводится по проволокѣ отъ концовъ ножницъ такъ, что вколо-

Frontispiece: Facsimile of the original description of the Eck fistula.

(Reproduced from Child, 1953)

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SUMMARY

The object of this work was to establish an experimental model in the rat to examine the relative roles of portal-systemic diversion and of impaired liver function in the pathogenesis of chronic portal-systemic encephalopathy (PSE). The problem with conventional end-to-side portacaval shunting, both clinically and in the experimental animal, is that in addition to producing total shunting of portal blood into the systemic circulation, the procedure itself leads to hepatocellular atrophy and impaired hepatic function. For this reason the new model of portacaval transposition (PCT) was developed in the rat. In this preparation total portal shunting takes place, but blood flow through the portal tracts of the liver is replaced by systemic blood from the inferior vena cava. Comparison of this model with the conventional portacaval shunt (PCS) should allow separation of the two phenomena of shunting and liver dysfunction. This is of some clinical importance, since it has been proposed that selection of patients or "tailoring" of portal diversion procedures on the basis of haemodynamic characteristics should lead to a lower incidence of chronic post-shunt encephalopathy, which remains the major clinical disadvantage of such operations.

It was first established (Chapter III) that PCT is attended by retention of a normal body growth pattern, little reduction in relative liver weight, and maintenance of normal hepatic morphology. This contrasts with PCS, following which there is impaired body growth and specific atrophy of the liver with marked histological changes. Since PCS rats were shown to have

reduced food intake, the specific nature of these findings was confirmed by using PCT and control animals which were pair-fed against PCS rats. It was also demonstrated that biochemical parameters of liver function were better conserved in the PCT preparation. It was further confirmed by use of radio-labelled Rose Bengal clearance that liver blood flow was in fact reduced in PCS rats and normal after PCT.

Sections prepared from the rapidly perfused brains of these animals were examined microscopically (Chapter IV). In both groups (and in a small number of control animals) it was possible to identify changes in the astroglial cells similar to those described by Alzheimer and characteristic of the brain in PSE in man and in experimental animals. However, these changes were significantly greater in the PCS rats than in the PCT rats, and in one experiment the abnormal cell count in PCT rats did not differ from control values. These results were also validated in a small group of pair-fed animals. This finding confirms the importance of hepatic dysfunction in the pathogenesis of central nervous system damage in these models, and suggests that maintenance of total hepatic blood flow even in the absence of direct perfusion by portal blood might confer protection against the development of PSE.

A characteristic pattern of plasma amino acids was seen following PCS, with reduction in the branched-chain amino acids valine, leucine and isoleucine, and elevation of the aromatic amino acids tyrosine and phenylalanine. This pattern, which characterizes chronic liver failure and PSE in man and experimental animals, was not seen after PCT (Chapter V).

Similar results were found in the pair-feeding experiment. Plasma insulin and glucagon levels were elevated to a similar degree after PCS and PCT. These results suggest that the hormone elevation seen as a result of portal-systemic diversion cannot entirely explain the amino acid imbalance, which appears to depend largely upon hepatic dysfunction in the PCS rats.

Changes in levels of glutamine, glutamate and tryptophan, substances known to be associated with central nervous system neurotransmission and possibly with glial function, were also examined (Chapter V). The differences observed did not achieve statistical significance, although the patterns seen amongst the groups were consistent with small but biologically important changes masked by metabolic compartmentation within the brain.

We conclude that the PCT model has proved valuable in separating the effects of portal diversion from those of hepatic dysfunction. The results confirm the vital role of normal liver function in protecting the brain against damage resulting from portal-systemic diversion, and suggest that liver function may be maintained close to normal even in the absence of direct portal perfusion if total liver blood flow is maintained. These findings are in keeping with the observation in man that a substantial compensatory increase in arterial flow following total portal diversion results in a lesser degree of encephalopathy. The establishment of the PCS/PCT model permits a further degree of refinement in future studies of the fundamental biochemical and cellular mechanisms of hepatic coma.

CHAPTER I. BACKGROUND

It is difficult to define portal systemic encephalopathy (PSE) in a universally satisfactory manner. However, PSE may be broadly described as a syndrome consisting of non-specific disturbances of mental state and of neuromuscular function, associated with liver disease and with portal-systemic shunting.

The impaired mental state, which is highly non-specific and may be found in any other form of progressive encephalopathy, ranges from the most trivial disturbances of personality, attention and mood to major behavioural disturbances and finally to coma. The neuromuscular disturbances in PSE are much more characteristic, although they may also be seen in other metabolic encephalopathies: the most familiar of these signs is asterixis, the classical "flapping tremor" which is almost the trademark of PSE.

In clinical practice this syndrome is most frequently associated with cirrhosis, but nevertheless it appears that it is the portal-systemic shunting associated with cirrhosis and not the cirrhosis itself which is the critical factor. While similar features may be found in a more florid form in coma associated with acute hepatic failure, it is likely that significant differences in pathogenesis exist between the acute and chronic forms of encephalopathy. It is largely the syndrome of chronic PSE with which this Thesis is concerned.

The importance of PSE to the surgeon lies in its existence as a complicating factor in the management of severe portal hypertension. It may occur in the unoperated patient with cirrhosis largely as a result of naturally occurring portal-systemic collaterals. Of greater importance, however, is the spectre of PSE which haunts the surgeon, and stands as the only major specific complication which compromises the results of portal-systemic shunting, the most effective treatment for bleeding varices. For several decades PSE has largely eluded both efforts at prediction in the individual patient and measures aimed at prevention or therapy. Moreover, despite an increasing literature its precise pathogenesis remains unclear.

Experimental investigation of chronic PSE has largely been based on the model of end-to-side portacaval anastomosis (PCS). Unfortunately, this preparation is not able to separate the effects of portal-systemic shunting from those of hepatocellular failure. It was against this background in 1972 that I began attempts to establish an alternative model which would allow further evaluation of these two closely inter-related factors. During the total time-span covered by this work (1972 to 1980) considerable progress has been made by other workers in this field, particularly with respect to the chemical mechanisms within the brain at the cellular and sub-cellular level. However, most work carried out on whole animal models has continued to make use of the PCS rat. The aims of the present studies were therefore:

1. To establish the model of portacaval transposition (PCT) in the rat, in order to allow separation of the effects of

portal-systemic shunting from those of hepatocellular deterioration which follows PCS, and to define carefully the fundamental characteristics of this new model.

2. To determine whether in the setting of well-maintained liver function portal-systemic diversion may produce a lesser degree of abnormality in the central nervous system. This situation may in some ways parallel the clinical position in which patients with better liver function are selected for shunting, or in which attempts are made to preserve hepatopetal flow by means of selective shunting procedures.

3. To examine some of the biochemical changes in plasma and in the brain which accompany portal-systemic shunting, and attempt to define which of these changes might have a potential causal relationship to PSE.

CHAPTER II. PORTAL-SYSTEMIC ENCEPHALOPATHY: HISTORICAL PERSPECTIVE

1. EXPERIMENTAL OBSERVATIONS

The term portal-systemic encephalopathy (PSE) was coined only 30 years ago by Professor Sheila Sherlock (Sherlock et al, 1954). However, this phenomenon or its equivalent has been observed for more than 100 years. The history of experimental portal-systemic shunting began with Nikolai Eck in 1877. Eck was a 29 year old military surgeon who performed total diversion of the portal blood into the vena cava in dogs. This procedure, the "Eck fistula", was carried out by suturing the walls of the two vessels side-to-side, and by introducing a specially designed scissors attached to a long thin silver wire with which it was possible to open a communication between the two vessels. The fistula was then made complete by ligation of the portal vein between the anastomosis and the liver. Eck had carried out the experiments to determine whether it would be possible to treat some cases of mechanical ascites by such a fistula. He felt he had demonstrated that

> "the main reason to doubt that such an operation can be carried out on human beings has been removed because it was established that the blood of the portal vein, without any danger to the body, could be diverted directly into the general circulation and this by means of a perfectly safe operation".

In fact, of the eight animals on which Eck operated one died in less than 24 hours, six survived from two to seven days, and one recovered completely and lived in the laboratory for 2.5 months, and then escaped "because of lack of attention". The cause of

death in the seven cases was peritonitis or strangulation of intestines and omentum.

A second seminal paper in the early history of PSE was published by Hahn, Massen, Nencki and Pavlov in 1893. These authors performed Eck fistula in 20 dogs. Following operation, the dogs suffered weight loss and hair loss, and when fed on a meat diet they developed the syndrome which became known as "meat intoxication", consisting of irritability and staggering, followed by convulsions and death if meat feeding was continued. If these dogs were fed on a meat-free diet then the syndrome was reversed. At autopsy liver atrophy and fatty infiltration were found. Pavlov noted that these events were minimized in cases in which there was shunt thrombosis or stenosis.

The syndrome of "meat intoxication" was reported once more in dogs by Balo (1932).

During the period 1920-1950, considerable attention was given to the mechanisms by which portal diversion led to atrophy of the liver, and the inter-relationship between this process and that of restoration of liver mass following resection. Rous and Larimore (1920) raised the possibility that portal venous blood was the source of "hepatotrophic" factors, while Mann (1940) put forward the hypothesis that the quantity rather than the quality of portal venous blood was the determining factor in maintenance of normal liver mass and function. This work led to the introduction in the dog of portacaval transposition by Child et al (1953), as a method for investigation of the hepatotrophic/flow controversy. This will be further discussed

in Chapter III.

The use of portal diversion models for investigation of PSE was not stimulated until the 1950s, after the recognition of PSE following portacaval shunting in man. Doyle (1967) in Edinburgh and Kyu and Cavanagh (1970) in London demonstrated that characteristic changes in the morphology of astrocytes in the CNS were produced by PCS in the rat. Similar changes had been demonstrated by Lapham (1961) following intraperitoneal injection of carbon tetrachloride. Since the early 1970s (when the work of this Thesis was commenced) there has been an explosion in the use of these and similar animal models to produce syndromes approximating to PSE in man, although even up to this time there remains no entirely satisfactory experimental model for the study of chronic PSE.

2. CLINICAL OBSERVATIONS

The proposed historical association of CNS abnormalities with chronic liver disease may be divided into the fanciful and the factual. The toxin clearing effect of the liver has been known since at least 1665, when Glisson inserted a quill into the portal vein of a human cadaver, and squeezed through this an ox bladder full of milk, which subsequently appeared in the vena cava. From this he concluded that one of the functions of the liver was to strain from the portal blood noxious substances originating in the gastrointestinal tract. Budd in 1845 stated "the role of the liver is to remove noxious substances from the blood stream". Sir Andrew Aguecheek in Shakespeare's Twelfth

Night is sometimes cited as the earliest documented sufferer of PSE, or Pavlov's "meat intoxication": "I am a great eater of meat and I believe it does great harm to my wit". However, the earliest conventional documentation of these syndromes may have been in the 1930s. Von Coulaert in Strasburg (1932) and Kirk in Denmark (1936) noted that administration of ammonium chloride or urea caused mental disturbance in cirrhotic patients. Monguio and Krause (1934) described mimicking of the encephalopathic effects of ammonia compounds by the ingestion of meat. Adams and Foley from Boston described in 1949 the syndrome now known as PSE in patients with cirrhosis, while in Norway in the same year Gaustad had described similar findings under the name "transient hepatargy". Gabuzda in 1952 showed that the same syndrome could be induced by means of ammonium-containing cation exchange resins.

The earliest recognition of the association between the Eck fistula and "episodic stupor" in man is usually credited to McDermott and Adams (1954), who also made the observation of elevated plasma ammonia levels. However, the historical credit for this observation rightly rests with the surgeon who performed the first portacaval shunt in man.

The French surgeon Vidal in 1903 presented a paper to the 16th Congress of French surgeons on the surgical treatment of ascites, which before this time had been managed by omentopexy. His report has been translated and published by Donovan (1978), and is a remarkable document. Forced to perform a direct end-to-side portacaval anastomosis because the omentum was unavailable in a young man with alcoholic cirrhosis, he gives a

lucid account of the operative procedure. After a smooth post-operative recovery, oral feeding was commenced on the fourteenth day. Vidal observed, however, that "all absorption of albuminoides provoked a very severe intoxication. Thus it is to hydrocarbons that one must turn for recourse." Vidal's patient suffered recurrent haematemesis at six weeks, but ultimately died of an acute septic episode.

Vidal stated very clearly the link between his own clinical observation and the experimental results of Eck and the other Russian physiologists, commenting on the intestinal production of

> "ammoniacal substances which are toxic for the organism when the liver is no longer there to guard against this danger and transform them into urea and other waste products....Even in limited amounts (albuminoides) produce sweats, muscular trembling, intense anxiety, cardiac arrhythmias - briefly a presentation characteristic of ammonia intoxication."

Several further attempts were made at clinical portal diversion in this early era. De Martel in 1910 reported an end-to-side portacaval shunt, but the patient died of acute renal failure in 24 hours. In the same year Villard and Tavernier attempted portal-systemic anastomoses using small vessels such as the ovarian and mesenteric veins, but were thwarted by anastomotic thromboses. Rosenstein in 1912 was the first to report side-to-side portacaval shunting for the treatment of ascites, in order to avoid the now recognized dangers of total diversion, and the same year brought the birth of the mesocaval shunt from a Russian surgeon, Bogoras.

The literature on this subject is then remarkably silent, until the presentation by Whipple (1945) to the American Surgical

Association ushered in the modern era of portal diversion. Following this, both McDermott and Adams (1954) and Hubbard (1958) reported PSE in patients with carcinoma of the head of pancreas, whose livers were grossly normal except for biliary obstruction. Pancreaticoduodenectomies were performed with resection of the portal vein, and in all four patients (two from each author) an end-to-side portacaval anastomosis was performed. Within a few weeks all had developed episodic hepatic encephalopathy, with malnutrition, fatty infiltration of the liver and hypoalbuminaemia. All died (tumour-free) between 4 and 20 months after operation. This rediscovery that man with a normal liver was susceptible to the same effects of portal diversion as those seen in Pavlov's dogs came at a time when the surgical management of portal hypertension with bleeding varices by means of portal diversion had become an established part of clinical practice (Blakemore and Lord, 1945; Blakemore, 1952).

Sherlock and her colleagues in 1954 coined the term "portal systemic encephalopathy", and also recognized the crucial relationship between the syndrome and impaired hepatocellular function. Ten years later the same group (Thompson et al, 1964) reported encephalopathy in 15% of non-cirrhotic patients undergoing portal diversion because of portal vein thrombosis. PSE and its association with the Eck fistula in man was a concept whose time had arrived.

CHAPTER III. THE DEVELOPMENT OF PCT IN THE RAT

1. BACKGROUND TO PORTAL DIVERSION MODELS

1.1 Portacaval shunt (PCS)

We have already seen (Chapter II) that the earliest model of portal diversion was that of Eck in 1877, and an encephalopathic syndrome following this procedure was recognized shortly thereafter (Hahn et al, 1893). The early uses of PCS for investigation of liver regeneration have also been described above. The widespread use of PCS as an experimental model was dependent upon the development of a reliable technique for its performance in the rat. The early development of these techniques has been reviewed by Lee et al (1974). The first method of PCS, using an indirect suturing technique, was described in 1946. However, the first sutured PCS using a microvascular surgical technique was reported in 1961 by Lee and Fisher, and this paper remains the standard reference for PCS in the rat. This work also described the principal features of the model, which subsequent research has largely confirmed: these features are outlined below.

Sequelae of PCS.

Weight loss is one of the most consistent consequences of PCS, with a loss of 5-30% of body weight over a period of two to four weeks after operation (Assal, Levrat, Cahn et al, 1971; Bismuth et al, 1963; Lee and Fisher, 1961). This effect appears

to be more marked in older animals (Kyu and Cavanagh, 1970). Liver weight is also significantly reduced following PCS. All of the above authors have demonstrated that the loss of liver weight following PCS exceeds the overall percentage loss of body weight, indicating that the liver undergoes specific atrophy. The morphological features described in association with this have ranged from early mitochondrial swelling and vacuolation with later proliferation of smooth endoplasmic reticulum and glycogen depletion (Oudea and Bismuth, 1965); increased cell density and marked sinusoidal collapse with centrilobular fatty change (Kyu and Cavanagh, 1970); degenerative infiltration of the hepatocytes with some evidence of binucleate cells (Henzel et al, 1963); or the complete absence of specific histological abnormalities (Assal et al, 1971). Enzyme defects have been reported by several groups following PCS. Drug metabolizing enzymes are reduced (Rubin et al, 1968) though this may be reversible by administration of phenobarbitone. Abnormalities in hepatic glutamine synthetase, uricase, and the enzymes of fatty acid metabolism have also been found (reviewed by Lee et al, 1974). Decreased levels of liver synthesized plasma proteins are also found in the chronic PCS rat (Kennan, 1964). Amino acid imbalance is also a constant sequela of PCS: this result may have specific importance and is considered in much greater detail below (Chapter V). Thus it has been well documented that PCS in the rat produces a variety of significant intrinsic hepatic abnormalities, most of which are believed to result from the loss of hepatic perfusion with hormone- and substrate-rich portal blood.

PSE following PCS in the rat

The readiness with which encephalopathy may be induced following PCS shows a marked species variation. Thus while it has been known for almost 100 years that dogs are rendered readily encephalopathic by meat feeding after PCS, it is relatively difficult to provoke an encephalopathic crisis in the PCS rat with a previously normal liver, and spontaneous coma is almost unknown. However, careful investigation reveals evidence of central nervous system damage in the majority of chronically shunted rats: this has been described in the form of spasticity or increased muscle tone or electroencephalographic abnormalities (Degos et al, 1969), loss of normal diurnal rhythms (Campbell et al, 1979), various electroencephalographic disturbances (Herz et al, 1972) and decreased startle reactivity (Warbritton et al, 1980). Addition of other manipulations to the PCS enhances these effects or makes them more readily detectable. Thus somnolence, apathy and muscular hypertonicity was consistently found by Degos and his colleagues (1969) in shunted carbon tetrachloride treated rats, and this finding correlated well with electroencephalographic abnormalities. Feeding of additional ammonia may produce encephalopathy (Hindfeld et al 1977). Addition of hepatic arterial ligation, usually carried out 24 hours or more after the initial PCS, produces complete hepatic devascularization. This has been used as a model of acute hepatic failure, and results in coma and the death of the animal (Mans, Saunders et al, 1979). Consideration of acute hepatic failure models is strictly beyond the scope of this work.

The ideal animal model for chronic PSE has not yet been achieved, and none of the available models correspond to all features of the clinical situation. Although spontaneous PSE is more subtle than in other species it is nevertheless well recognized that the histological central nervous system lesions associated with PSE in man and other animals are consistently reproduced in the PCS rat (Doyle, 1967; Cavanagh et al, 1972). More detailed consideration is given to these changes in Chapter IV below. For practical purposes, PCS in the rat has achieved widespread acceptance despite the possible importance of species differences (Cavanagh, 1974). The principal defect of the PCS model in relation to PSE is that in addition to diverting portal blood into the systemic circulation, bypassing the hepatic filtration and detoxification functions, the liver is also deprived of approximately half of its normal tissue perfusion (Heer et al, 1963). This is followed by an inevitable sequence of liver atrophy and impaired hepatocellular function, as described above. A model which would allow systemic diversion of portal blood without these complicating effects would be more ideally suited to investigation of the pathogenesis of PSE.

1.2 Introduction of Portacaval Transposition

In 1953 Child and his associates introduced the preparation of PCT in the dog. In this operation all the portal blood was drained systemically by end-to-end suture of the divided portal vein to the divided vena cava. The input to the portal tracts of the liver was replaced with systemic venous blood by end-to-end anastomosis of the portal vein and distal vena cava. This model was introduced to elucidate the mechanism of regenerative

hyperplasia in the liver, and specifically to determine the relative importance of the volume of blood flow (Mann, 1940) and of hepatotrophic substances in portal blood (Rous and Larimore, 1920). The work of Child provided at that time strong suggestive evidence in favour of the flow hypothesis and against the hepatotrophic concept. (Further work over the last 20 years has cast considerable doubt upon the conclusions from this study, and this will be discussed further below: nevertheless the use of the model was conceptually valuable, and opened the way to a useful new line of investigation.) Transposition was also performed in cirrhotic dogs in 1958 (Owsley, Harper et al, 1958). Starzl and his colleagues (1961) described a modification of the PCT operation using a temporary side-to-side mesocaval shunt and a caval-jugular bypass, which reduced the mortality of the procedure in the dog. Ono et al (1968) in McDermott's laboratory used a porta-renal transposition in dogs, a model which was the fore-runner of the first 'transposition' preparation to emerge in the rat (see below).

The observation that PCT produced deglycogenation of the liver (Sexton et al, 1964) led to its clinical use by Starzl and his colleagues (Starzl, Marchioro et al, 1965) and by Riddell and his colleagues (1966) in England for the treatment of glycogen storage disease. The first of the patients so treated by Starzl's group, a child with type III glycogen storage disease, remains alive almost 20 years after PCT (Starzl et al, 1983). Riddell's patient has apparently also enjoyed a long survival. Starzl's second patient died two days after PCT because of hepatic congestion consequent on the high volume flow of the

inferior vena cava. Since this time Starzl's group have reported the use of conventional end-to-side PCS in a further eight patients with glycogen storage disease, of whom seven are alive with follow-up of between 6 and 11 years. Only one of these patients developed significant signs of chronic PSE: this 16 year old girl was treated by successful orthotopic liver transplant 8 years after the original PCS (Starzl et al, 1983). This observation is in contrast to the reports by Mikkelsen and his colleagues (1965) and by Voorhees (1973) demonstrating encephalopathy or subtle psychological and psychiatric disturbances following PCS for extrahepatic portal block. These important results will be discussed further below.

The model of PCT in the dog has been used sporadically for physiological research in a variety of other areas including gastric acid secretion (Clarke et al, 1958; Schafmeyer et al, 1979), hepatic physiology (Silen et al, 1956, 1957; Summers et al, 1956), and renal vascular hypertension (Sweeney et al, 1962; Palmer 1963; Valcin et al, 1972).

In essence, PCT in the dog has been shown to result in better maintenance of liver function (assessed by hepatic enzymes and plasma proteins) and of body weight and liver weight, compared with animals subjected to conventional PCS. One consistent residual defect appeares to be impaired clearance of ammonia following PCT (Silen et al, 1957; Owsley, Goin et al, 1958). It has become clear (both from the present work and from other results published during the time of these studies) that replacement of the inflow to the portal tracts by systemic venous blood cannot maintain entirely normal liver function or even

normal liver mass Nevertheless, large differences from the sequelae of PCS are apparent, and are of sufficient magnitude to validate the use of the model.

Development of PCT in the rat

The first report of PCT in the rat was that of LeCompte and his colleagues in Bismuth's laboratory in France (LeCompte et al, 1973: first reported in abstract form in 1970) These workers used the left renal vein following nephrectomy for anastomosis to the portal vein. Few observations were reported in their animals, but these confirmed the finding in dogs with PCT of rapid restoration of body weight and return to a normal growth curve within a few days of operation.

Objectives of the present PCT programme

It was clear from the foregoing studies that comparison of PCT and PCS in the rat would be of value for studies of PSE and of liver regeneration. Our initial technical objective was to produce PCT in rats by the same technique as that used in the dog, namely direct end-to-end anastomoses of the vena cava and portal vein. It was then clearly important to define the behaviour of these animals in comparison with PCS rats, and in particular to confirm differences in growth rate and in the degree of liver atrophy and histological damage, and to establish differences in biochemical liver function. All of these investigations would be necessary as an initial step in demonstrating the production of a model of total portal-systemic diversion which could maintain liver mass and function significantly better than conventional PCS. The results of these

fundamental studies are the subject of this chapter.

2. DIRECT PORTACAVAL TRANSPOSITION IN THE RAT

The animals used for these studies were male Sprague-Dawley or Long Evans rats, the strains in regular use at the two centres in which the studies took place (see Section 3 below). Growing rats within the weight range 250-390 g at the time of operation were used in all the experiments, and within each individual experiment the weights of the different groups of rats were as closely matched as possible. Animals were not fasted prior to operation, and post-operatively they were housed in groups of four to a cage, and allowed free access to water and a standard pellet diet. Housing and feeding details for pair-feeding experiments are described below. All animals were housed in conditions of controlled temperature, with artificial lighting arranged in 12 hour light/dark cycles.

SURGICAL TECHNIQUE

The operations were carried out in clean but not sterile conditions, under open ether anaesthesia. The rat was taped down in a supine position on a board, and the abdomen opened from the xiphisternum to the pubis through a midline incision. The anterior two lobes of the liver were gently mobilised by division of the suspensory ligaments, and delivered on to the thorax where they were kept moist under a gauze swab. The inferior vena cava was then stripped of its peritoneal and adventitial covering from the inferior border of the liver to below the right renal vein. The portal vein was carefully mobilised between the porta hepatis and the termination of the splenic vein, with ligation and division of the gastroduodenal (right gastric) vein between 5/0

silk ligatures. Great care was taken during this part of the dissection to avoid damage to the hepatic artery, since trauma or subsequent spasm of this artery may lead to subsequent hepatic ischaemia, and sometimes to hepatic necrosis and death of the animal.

The procedure from this point depended upon whether portacaval transposition (PCT), portacaval shunt (PCS), or control (sham) procedure was to be carried out.

(a) Portacaval Transposition

(See also illustrations in Ryan et al 1974, Appendix C.)

Small bulldog clamps were applied to the vena cava above the right renal vein and below the inferior surface of the liver, and the vessel divided between these clamps. The portal vein was similarly divided between bulldog clamps and the open ends of the vessels flushed with saline from a 1 ml syringe. The remainder of the operation was carried out using magnification by means of a Zeiss operating microscope with coaxial illumination, using approximately 10 x magnification. Two stay sutures of 7/0 silk were applied attaching the distal end of the portal vein to the proximal end of the inferior vena cava, taking care not to tear or twist the veins. The anastomosis was completed by means of a continuous suture of 7/0 silk, following which the clamps were removed. The anastomosis was "massaged" at this stage using a cotton bud in order to disperse any small clots at the anastomotic line. During the time of portal venous occlusion (less than 15 minutes) the intestines, which were retracted to the left of the animal, became congested, but returned briskly to

a normal pink colour on release of the clamps.

A second anastomosis between the distal inferior vena cava and proximal portal vein was then carried out in a similar fashion, using stay sutures and a continuous suture of 7/0 silk. This anastomosis lies comfortably in front of the first anastomosis. On removal of the clamps there may appear to be a degree of constriction at the anastomosis due to increased pressure within the distal vena cava. However, this appearance is transient and is never found at subsequent laparotomy. Moreover, further work from this laboratory in which the inferior vena caval pressure was measured post-operatively demonstrated that there is no prolonged elevation of caval pressure (Ryan et al, 1978).

Following final inspection of the anastomoses the intestines and liver lobes were carefully returned to the abdomen and the wound closed with a single layer of continuous 3/0 chromic catgut to the muscle, and either a further layer of chromic catgut or metal clips to the skin.

(b) End-to-Side Portacaval Shunt

Following mobilisation of the vena cava and portal vein, a curved clamp was applied to the vena cava just above the level of the right renal vein, and a 4-5 mm venotomy made in the anterior wall. A bulldog clip was applied to the portal vein close to its origin, and the portal vein ligated using 5/0 silk as close to the porta hepatis as possible. The vein was then divided just below this ligature and stay sutures placed between the cut end of the portal vein and the inferior vena cava and end-to-side

anastomosis constructed with continuous 6/0 silk. The time taken to the point of releasing the clamps and opening the anastomosis was less than 15 minutes. The operation was concluded in the same way as for PCT above.

(c) Sham Operation (control animals)

Following mobilization of the vessels as described in (a) above, clamps were applied to the inferior vena cava and to the portal vein, but no further manipulation was carried out. The clamps were removed after 15 minutes and the operation concluded in the same way as in (a) and (b) above.

Animals were kept individually in a warming box or a cage beneath an anglepoise lamp until recovery, which took place within a few minutes of cessation of anaesthesia. They were returned to their normal housing after a further 30-60 minutes.

3. EXPERIMENTAL DESIGN AND EXPERIMENTAL GROUPS

3.1 Centres Involved

Since this work was carried out in two centres, there has been an inevitable overlap in the experiments used to define the PCT model.

During the years 1972-1975 the work was carried out in the St. Mungo Department of Surgery at Glasgow Royal Infirmary, where the rats in use were male rats of the Sprague-Dawley strain. During this time the technique of operation was developed as described above, and general observations were made as to the effects on body weight, liver weight, liver histology and hepatic enzymes. In addition studies were carried out on changes in serum proteins and the effect of PCT on liver regeneration following partial hepatectomy, and the results of these studies are described below. Finally, a small pilot study on the metabolic effects observed in pair-fed animals was carried out.

During the period 1978-1979 further work was undertaken in the laboratories of the Department of Surgery of the University of Cape Town at Groote Schuur Hospital. Although the major studies performed in this centre related to amino acid and hormone changes in the plasma and to histological and biochemical changes in the brain, some of the fundamental studies carried out in Glasgow were repeated in Cape Town because of the different strain of rats in use at that centre (Long Evans), in order to ensure consistency of the data. For this reason there is some duplication of results from the two centres, and wherever this might be considered important the duplication will be pointed out

in the text and in the results. However, since in fact no fundamental differences were observed between the two centres, wherever appropriate the results from the two series of experiments have been combined.

3.2 Chronic and Acute Studies

Most of the studies fall into the category of "chronic" experiments, in which rats were observed over a period of 5-12 weeks following the procedures. In the experiments in which rats were fed ad lib, every rat was removed from its cage weekly, and under a brief ether anaesthesia 1 ml of whole blood was withdrawn from the retro-orbital venous plexus by means of a Pasteur pipette. There was no morbidity associated with this procedure. Following this the animal was weighed and returned to its cage. In the pair-feeding experiments, every animal was removed from its cage and weighed daily, but the animals were not anaesthetised and weekly blood samples were not obtained.

The "acute" or short-term experiments were designed to examine the immediate effects of the procedures on plasma biochemistry. The objective was to obtain blood samples at 6, 12, 18, 24, 42, 48, 66 and 72 hours after the operative procedure. In order to minimize blood sampling from individual rats, each experimental group in these acute studies was divided into subgroups which were bled at alternate time points. (For purposes of analysis the results obtained at 66 and 72 hours were regarded as having come from one group, and the values pooled.) In the Glasgow studies, one subgroup was bled at 6,12,18, and 24 hours (at which time the animals were sacrificed), and a second

subgroup was bled at 1,3,5, and 7 days, in order to extend the period of study for the acute experiments. In an additional experiment, several extra control groups were used for biochemical studies: in order to simplify description at this stage, description of the groups used in this single experiment is deferred until presentation of the results in Section 4.1 below.

3.3 Controlled Feeding Studies

Two pair-feeding studies were carried out, one in Glasgow and one in Cape Town. In each case animals were housed in individual metabolic cages, and the food used was the same pellet food as in the ad lib feeding groups, ground to a powder and administered in a feeding compartment in order to avoid food losses through the wire cage floor. The experiments were designed to control the feeding of PCT and control animals against the ad lib intake of PCS rats. Rats were acclimatized to the metabolic cages for several days prior to operation. The PCS rats underwent operation before the other groups, and were allowed free access to the ground food and water. Weight-matched animals were paired with indvidual PCS rats, and selected to undergo either PCT or control operation. Each PCT and control animal was then allowed the same quantity of food consumed on the equivalent post-operative day by its PCS pair. Animals for both of these experiments were handled and weighed daily. The Glasgow experiment continued for six weeks, and the Cape Town experiment for ten. In the Glasgow study daily nitrogen intake and output was estimated by total collection of urine from each rat, and estimation of nitrogen content of this and of the diet by the

Kjeldahl method (see Appendix A).

3.4 Sacrifice Procedures

At the time of sacrifice animals were anaesthetized with ether, and a laparotomy performed. At this procedure particular attention was paid to the presence or absence of intra-abdominal adhesions, particularly those which might carry collateral blood supply to the liver following portacaval shunting. In practice, no such vascular adhesions were found. Patency of the anastomoses was confirmed by dissection and inspection. Blood was withdrawn, usually by formal cannulation of the abdominal aorta with a polyethylene cannula. Powdered aprotinin (Trasylol, Bayer and Co. Ltd.) was added to aliquots of the blood when appropriate as a preservative for hormone estimations. In some cases *in situ* brain perfusion was undertaken at this stage, and this is described below (Chapter IV). In the other cases the whole liver was excised, blotted and weighed at this point. Dry liver weight was estimated (in the Cape Town studies) by drying excised livers in a warm air oven with daily weighing until a constant weight was attained.

3.5 Laboratory Methods

a) Biochemical

(i) Proteins (Glasgow): Assays were performed on separated serum, stored for up to three months at -20°C. Serum albumin, IgG, and α_1-globulin were measured by radial immunodiffusion assay on plates containing monospecific antiserum raised in rabbits as previously described (Goudie et al, 1966) and measured by the Mancini technique (Mancini et al, 1965).

(ii) Serum enzymes: In the Glasgow series aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), creatine kinase (CK), and lactate dehydrogenase (LDH) were measured on separated serum using a Beckmann autoanalyzer system. In Cape Town only AST was measured, using a standard manual enzymatic method (Karmen et al, 1955) on a Beckmann spectrophotometer.

(iii) Glucose (Glasgow): This was measured on separated plasma using a Beckman glucose autoanalyzer.

(iv) Nitrogen (Glasgow): This was measured in food and urine by the Kjeldahl method (Appendix A).

b) Histological methods

All liver biopsies were cut from the median lobe of the liver immediately after removal and weighing. Material for light microscopy was fixed and stored in buffered formalin, and embedded in paraffin and processed in the routine manner. In

some cases material was also obtained for electron microscopy. Finely diced tissue was fixed in glutaraldehyde in phosphate buffer for 24 hours followed by storage in phosphate buffer until processed. The specimens were replaced in glutaraldehyde and phosphate overnight and then processed using phosphate buffer, post-osmication, uranyl acetate block staining and alcohol dehydration, and embedded in Spurr's resin. Thin sections were cut on an LKB ultramicrotome, mounted on copper grids and stained with uranyl acetate and lead citrate.

4. RESULTS

4.1 GENERAL OBSERVATIONS IN ACUTE AND CHRONIC STUDIES

The operation of PCS is now fairly well established as a routine research procedure, and after a few hours' training can be readily accomplished with negligible mortality. PCT is a more exacting procedure, but with skill and practice a mortality rate of less than 10% can be achieved. Most deaths which occur are due to technical errors, such as damage to the hepatic artery during dissection or inadequate anastomosis, and these deaths occur within the first 24 hours. Animals in this category have not been included in the present studies. The total number of animals used, and their distribution between the two centres and the various experimental groups, is shown in Table 3.1. These were the animals used in the general experiments described, and exclude those used for hepatic Rose Bengal clearance studies, liver regeneration experiments, studies of immunoglobulins and immunological tolerance, and ovarian implant studies. These additional studies are referred to below (Sections 4.3 to 4.5).

Within a few days of operation it became possible to distinguish between the behaviour of PCS animals and the other groups, although no differences were observed between PCT and control rats. PCS rats rapidly developed an ill-groomed appearance, and were less active in their cages and more irritable on handling. However, although detailed behavioural studies were not performed, there was no evidence of stupor or coma, nor other signs of overt hepatic encephalopathy.

TABLE 3.1

Animals used in Glasgow and Cape Town studies

	Glasgow			Cape Town			Total		
	C	PCT	PCS	C	PCT	PCS	C	PCT	PCS
Acute experiments	6	6	6	(1) 12 (2) 6	12 6	12 8	24	24	26
Chronic experiments	20	11	11	(1) 15 (2) 8	10 8	10 9	43	29	30
Pair-feeding experiments	3	4	4	3	6	6	6	10	10
Totals	29	21	21	44	42	45	73	63	66

Total rats = 202

Note: total excludes rats used in Rose Bengal clearance studies and in Glasgow pair-feeding and metabolic studies.

Body weight (Figure 3.1)

By the end of the first week after operation, PCS animals had lost up to 15% of their initial body weight. PCT rats also lost a little weight in the first week, though only one had lost more than 10%. Furthermore, the weight loss was sustained in the PCS rats, but rapidly regained in the PCT animals, which thereafter followed a growth curve almost indistinguishable from control animals. The mean maximum weight loss in PCS rats was 19.1% of body weight (± 6.5% SD). No PCS rat had regained its pre-operative weight before the fifth week amd some had not done so by 10-12 weeks. For PCT rats the mean maximum weight loss was 4.5% (± 4.4 SD). Moreover, there was only one rat in the PCT group which had not passed its pre-operative weight by the second week. The marked weight loss following PCS has been well described previously, and subsequent studies have demonstrated that this is largely due to a diminished food consumption in these animals. The results of our own pair-feeding studies, which confirm these observations, are described in Section 4.2 below.

The early pilot studies in Glasgow showed that during a post-operative follow-up period of five weeks, older and heavier (340-505 g) PCT animals lost more weight post-operatively than the younger and lighter (270-325 g) rats. These older animals also showed a slower recovery of their body weight, although they remained heavier in relation to their pre-operative weight than animals with PCS by the time of sacrifice (see Ryan et al, 1974 in Appendix C). These observations remain unconfirmed, since in the subsequent studies reported in this Thesis younger rats in

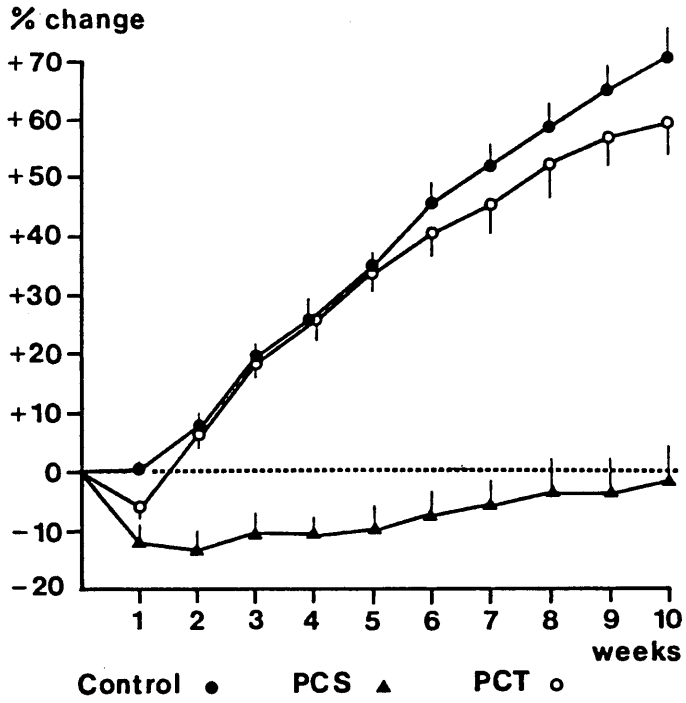

Figure 3.1

Percentage change from pre-operative body weight in ad lib fed rats 1 to 10 weeks after operation.
Each point is the mean of one group.
Error bars show standard errors of the mean.

the lower operative weight range were consistently used.

A tabulation of body weight changes in the chronic Cape Town studies is given in Appendix B, Table 1.

Liver weight

The wet liver weight (and in the Cape Town experiments the dry liver weight) was determined at the time of sacrifice in all "chronic" studies. It has previously been established that in normal young growing rats the weight of the liver is a fairly constant proportion of the total body weight (Brues et al, 1936). and this was confirmed in studies in our own laboratories. Since the changes in body weight at the conclusion of the chronic experiments ranged from + 90% (control) to - 21% (PCS), absolute liver weight gives a misleading estimate of the relationship between liver mass and body size. The relationship between liver mass and body mass at sacrifice in control, PCS and PCT rats is shown in Figs B1-B3 in Appendix B: these figures show that this relationship is maintained in the present series of experiments, although of course the parameters of the regression equation are different for each experimental group. All comparisons have therefore been made between values of "relative liver weight", which is the liver weight expressed as a percentage of the body weight at the time of sacrifice. These results are shown in Table 3.2, where the values for liver weight are expressed both in absolute (Grams) and relative terms. Figure 3.2 shows graphically the values of wet and dry relative liver weights at 10-12 weeks in ad lib fed rats in the Cape Town series.

TABLE 3.2

Liver weights and body weights at sacrifice in Glasgow and Cape Town studies

All values are mean ± 1 SEm

Rel. liver weight = liver wt x 100/body weight (see text)

		Control	PCT	PCS
Glasgow (5 weeks)	n	20	13	11
	mean body wt. (g)	396 ± 8.4	331 ± 12.1	264 ± 14.1
	mean liver wt. (g)	11.9 ± 0.31	9.2 ± 0.36	5.6 ± 0.39
	mean rel. liver wt. (%)	3.00 ± 0.04	2.78 ± 1.13	2.09 ± 0.05
Cape Town (10–12 wks) ad lib fed	n	14	15	14
	mean body wt. (g)	516 ± 13.9	547 ± 13.1	339 ± 13.1
	mean liver wt. (g)	16.7 ± 0.6	15.2 ± 0.72	7.0 ± 0.46
	mean rel. liver wt. (%)	3.24 ± 0.08	2.77 ± 0.09	2.07 ± 0.10
	mean rel. dry liver wt. (%)	1.10 ± 0.06 (n = 5)	0.94 ± 0.04 (n = 8)	0.67 ± 0.05 (n = 6)
	wet:dry weight ratio	2.78 ± 0.09	2.94 ± 0.05	3.02 ± 0.10
Cape Town (13 wks) pair-fed	n	3	6	6
	mean body wt. (g)	334 ± 9.0	336 ± 11.8	337 ± 17.8
	mean liver wt. (g)	8.5 ± 0.17	8.2 ± 0.29	6.25 ± 0.67
	mean rel. liver wt. (%)	2.55 ± 0.06	2.47 ± 0.04	1.83 ± 0.05
	mean rel. dry liver wt. (%)	0.72 ± 0.02	0.74 ± 0.01	0.58 ± 0.01
	wet:dry weight ratio	3.56 ± 0.21	3.31 ± 0.16	3.17 ± 0.12

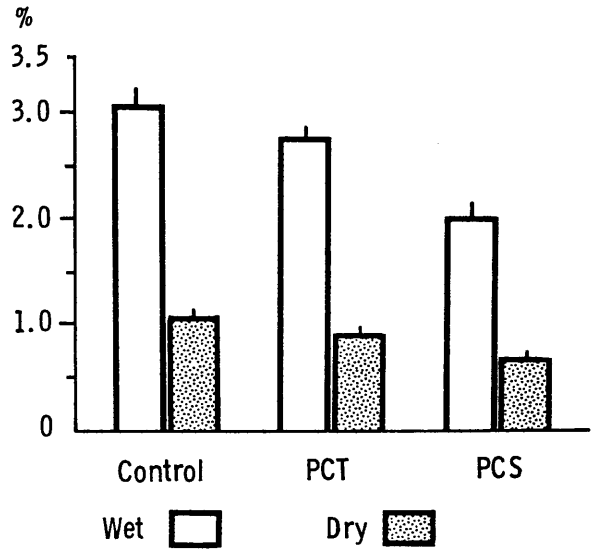

Figure 3.2

Relative wet and dry liver weights (liver weight as percentage of body weight at sacrifice) 10 weeks after operation in ad lib fed rats.

Each bar shows the mean relative liver weight for rats of one group. Error bars show standard errors of the mean.

TABLE 3.3

Relative liver weight (%body wt) at 10 weeks in ad lib fed animals

	CONTROL	PTC	PCS
	3.54	3.47	2.67
	3.53	2.98	2.02
	3.33	2.44	2.29
	3.34	2.94	2.09
	3.17	2.41	1.47
	2.79	2.31	1.75
	3.67	2.88	2.46
	3.24	2.50	2.18
	3.50	2.64	1.95
	3.00	2.88	1.80
	3.23	2.92	2.69
	3.10	2.50	2.13
	2.63	2.77	1.74
	3.29	3.31	1.70
		2.57	
		2.77	
Mean	3.24	2.77	2.07
SE_m	± 0.08	± 0.090	± 0.10

One-way ANOVAR table for above data

Source of variation	Sum of squares	Deg of freedom	Mean square	Variance ratio
Between groups	8.76	2	4.380	32.44 ***
Residual	5.41	40	0.135	

Detailed comparisons

Control vs PCS	9.629	1	9.629	68.66 ***
Control vs PCT	1.506	1	1.506	11.15 ***
PCT vs PCS	3.557	1	3.557	26.35 ***

*** $p < 0.001$

The figures are very similar for the Glasgow and Cape Town studies. There is a significant reduction in liver weight in the PCT animals by 5 weeks after surgery, which persists at 10-12 weeks. However, the reduction in liver weight is much more marked in PCS animals, and a significant difference exists between PCS and PCT rats at 5 weeks, and persists at 10-12 weeks. A table for one-way analysis of variance applied to these results is given in Table 3.3.

In the group of animals from Cape Town in which dry liver weight was available as well as wet weight, similar changes were observed (Table 3.2). There was a small but significant reduction in dry liver weight in PCT animals compared with control, but once again a much more marked reduction was seen in PCS animals. The ratio of wet to dry liver weight (also shown in Table 3.2) did not differ significantly amongst the groups.

Light microscopy of the liver

Sections of liver obtained at sacrifice from groups of animals at three days and at 10-12 weeks in the Cape Town study were encoded and examined "blind" and systematically graded by a senior histopathologist (Dr. JAH Campbell). The overall results of this assessment are shown in Table 3.4. At 72 hours the only changes seen in the control and PCT animals were of mild to moderate fatty change with fat deposits which did not distort the nuclei: these changes were seen in 3/11 control animals and 1/8 PCT rats. However, in the PCS group at 72 hours, 8/11 animals showed central necrosis with infiltration of fibroblasts, lymphocytes, and plasma cells. In the 2 animals most severely

affected this was accompanied by marked fatty change and bridging necrosis. In 2 further animals there was mild fatty change present centrally, and only one PCS rat had a normal appearance. Mitotic figures were numerous in some PCS animals.

At 10-12 weeks there was a mild degree of fatty change in the central- and mid-zones in only 1/14 control and 1/9 PCT rats. By contrast, only 3/10 PCS rats showed no major histological changes. Two PCS rats showed mild peripheral fatty infiltration, and in 5 fatty infiltration was moderately severe, usually distorting the nuclei and associated occasionally with necrotic cells. These changes are illustrated in Figures 3.3-3.8.

In order to assess the degree of hepatocyte atrophy in the chronic studies, the technique of Kyu and Cavanagh (1970) was used. This technique measures the nuclear density by counting the number of nuclei per high power field for each animal. Using a measuring graticule to ensure counting of identical cross-sectional areas of tissue, counts were obtained separately for the central (periportal) and peripheral zones of the liver lobule. The results (expressed as nuclei per high power field) are shown in Table 3.5. No significant differences were observed between the nuclear counts of control and PCT animals, in either the peripheral or central zones. PCS rats showed a significantly higher nuclear count in both the peripheral and central zones than either control or PCT animals. In addition to the differences between groups, the nuclear density in the peripheral zone in control and PCT animals was significantly higher than that in the central zone. However, following PCS this difference

TABLE 3.4

Histological changes in rat liver on light microscopy

	72 hours	10 weeks
Control	8 Normal 1 Minimal fatty change 2 Moderate fatty change	13 Normal 1 Mild fatty change
PCT	7 Normal 1 Mild fatty change	8 Normal 1 Mild fatty change
PCS	1 Normal 6 Healed central necrosis 2 Bridging necrosis + fatty change 2 Mild fatty change	3 Normal 5 Moderate to severe fatty change with cell necrosis 2 Mild fatty change

TABLE 3.5

Nuclear density in Long Evans rats 10-12 weeks after operation. The number of hepatocyte nuclei in a high-power field is shown for each animal, a separate count being made for peripheral and central zones.

C O N T R O L			P C S			P C T		
Central	Peripheral	Total	Central	Peripheral	Total	Central	Peripheral	Total
18	22	40	49	49	98	19	25	44
23	31	54	61	58	119	28	32	60
28	31	59	51	50	101	13	25	38
24	26	50	40	40	80	34	51	85
39	39	78	90	69	159	23	33	56
25	30	55	56	70	126	27	31	58
26	28	55	41	39	80	28	30	58
26	39	65	32	33	65	30	30	60
31	31	62	27	46	73			
25	34	59						
mean 26.5 +	31.1	57.6	*49.7	**50.4	100.1	25.3 +	32.1	57.4
SEm 1.7	1.7	3.2	6.2	4.3	10.1	2.4	2.9	4.9

*Differs from control and from PCT, $p < 0.005$
**Differs from control and from PCT, $p < 0.001$
+Central and peripheral counts differ, $p < 0.05$ (Student's unpaired t-test)

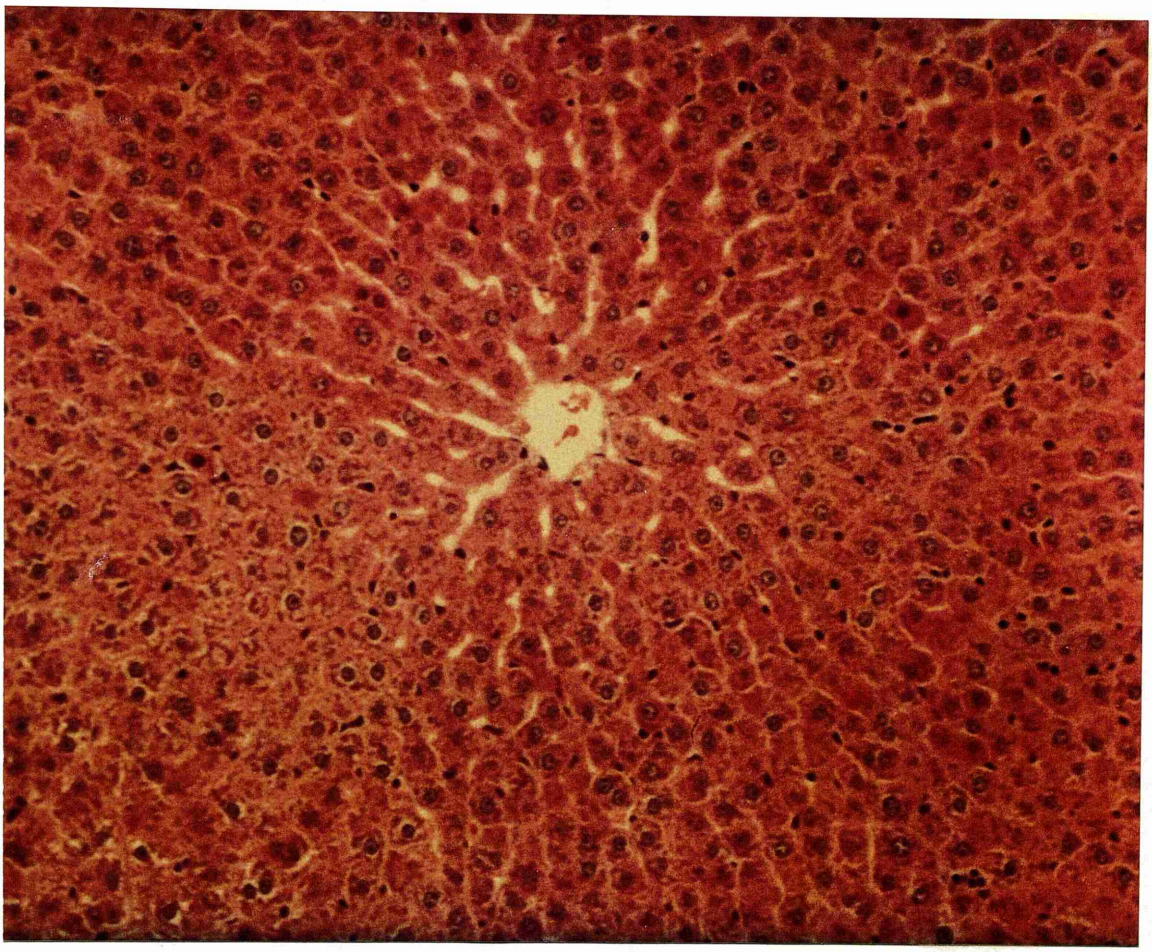

Figure 3.3

Light microscopic appearance of liver 72 hours after control operation.

Haematoxylin and eosin. Magnification X 400.

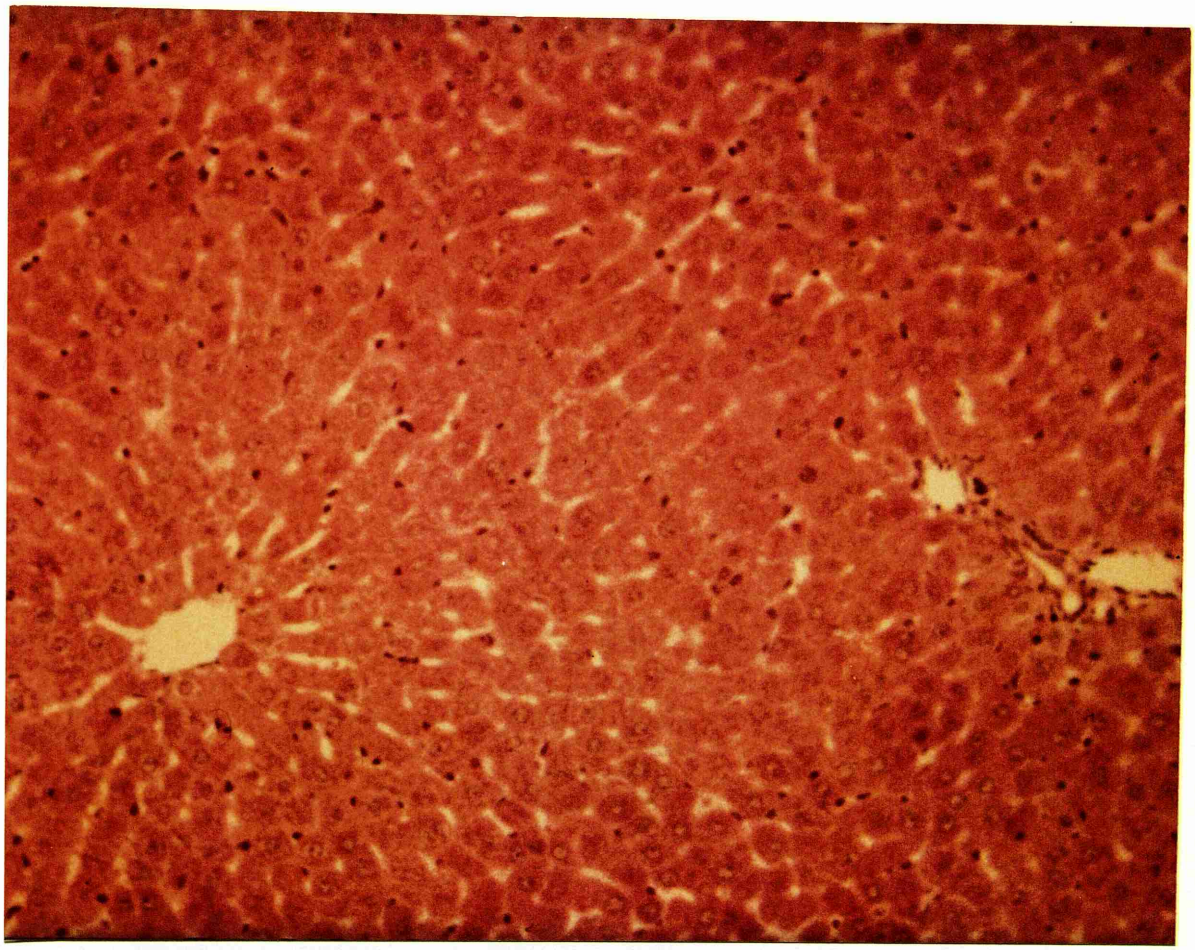

Figure 3.4

Light microscopic appearance of liver 72 hours after portacaval transposition. Haematoxylin and eosin. Magnification X 400.

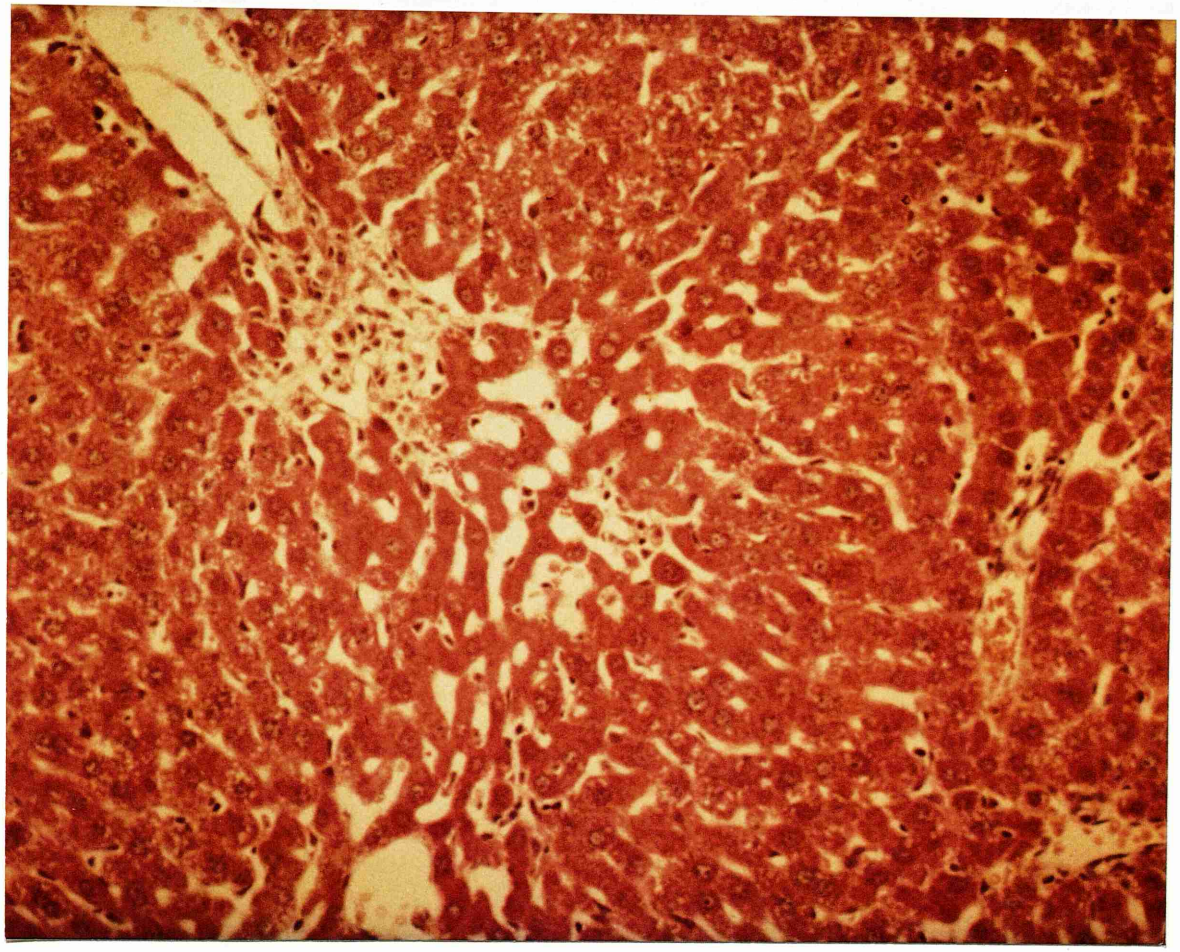

Figure 3.5

Light microscopic appearance of liver 72 hours after portacaval shunt. Note the healing central necrosis.

Haematoxylin and eosin. Magnification X 400.

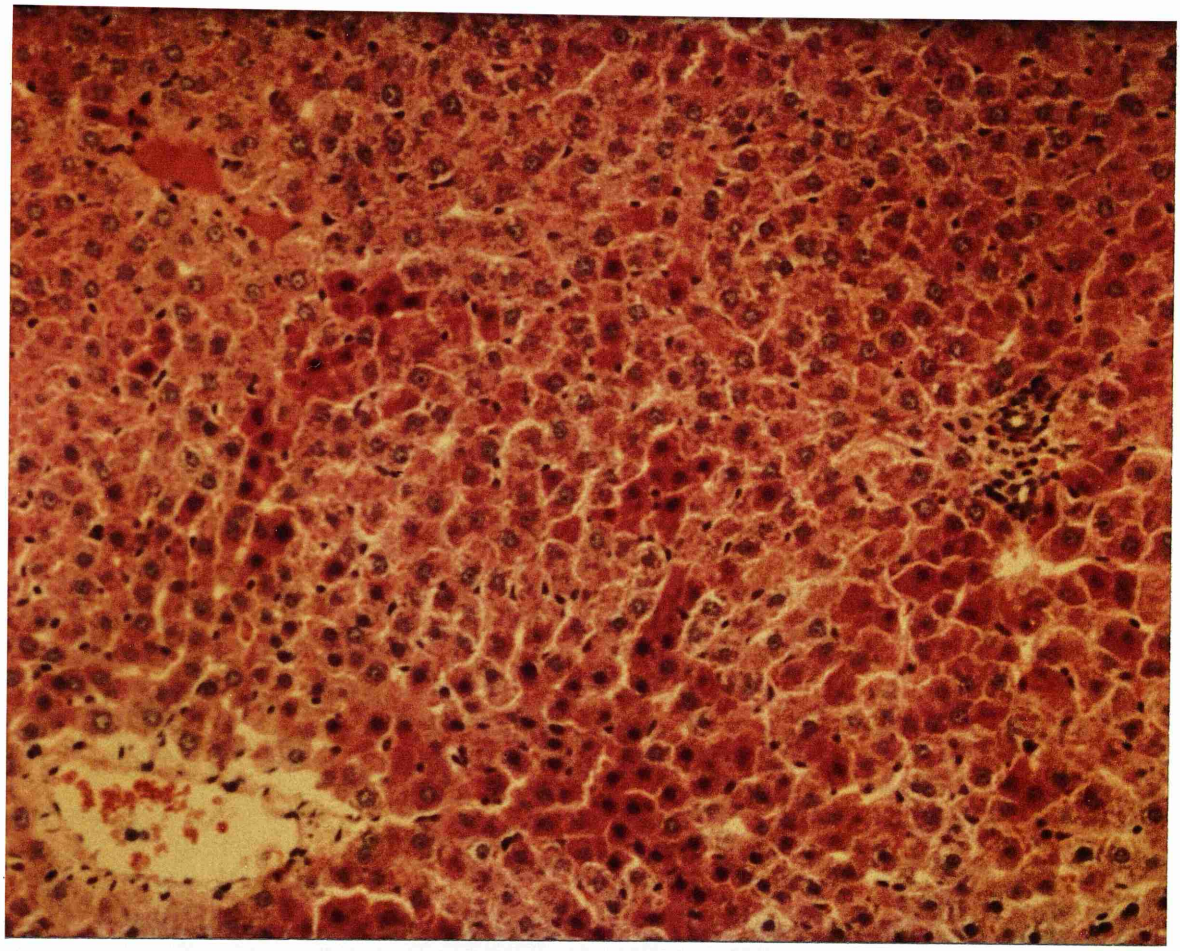

Figure 3.6

Light microscopic appearance of liver 10 weeks after portacaval shunt.

Haematoxylin and eosin. Magnification X 400.

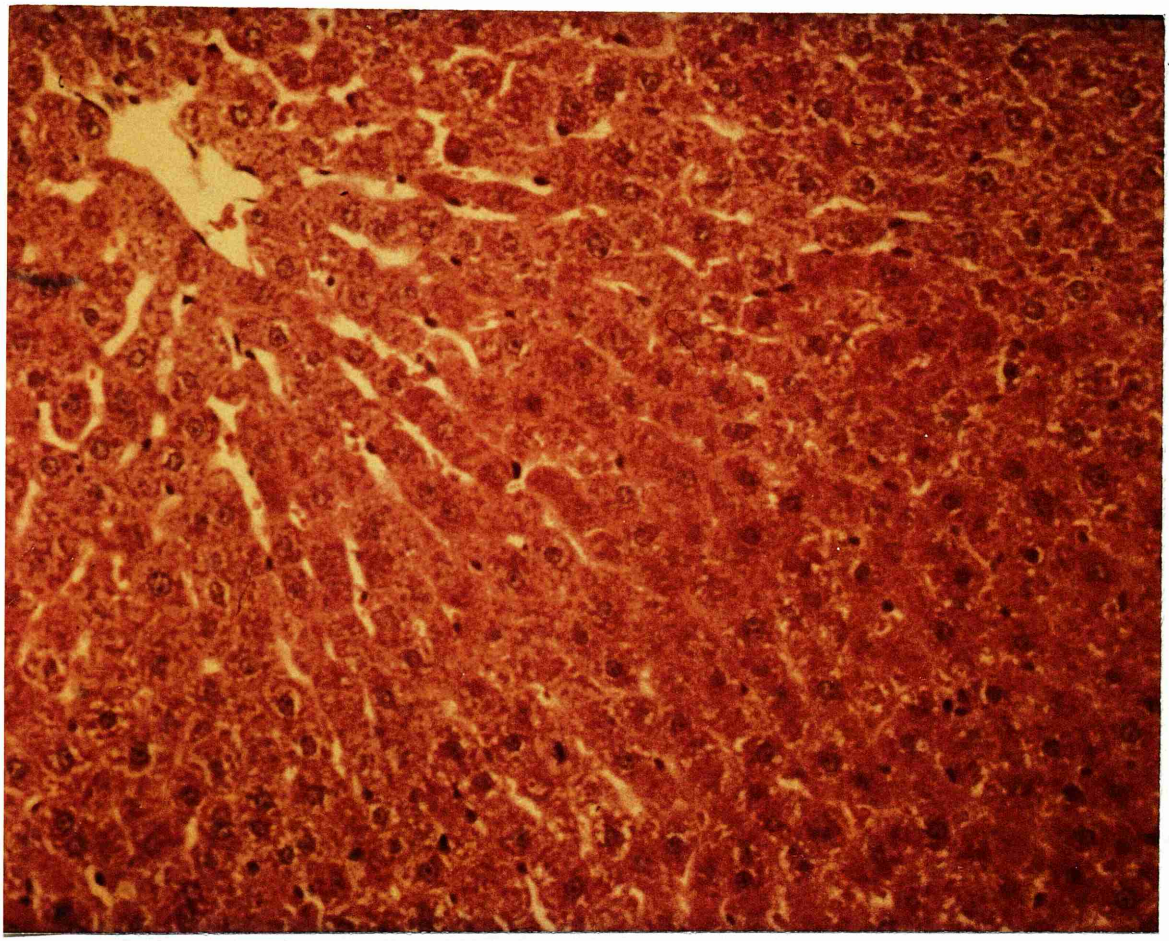

Figure 3.7

Light microscopic appearance of liver 10 weeks after portacaval transposition.

Haematoxylin and eosin. Magnification X 400.

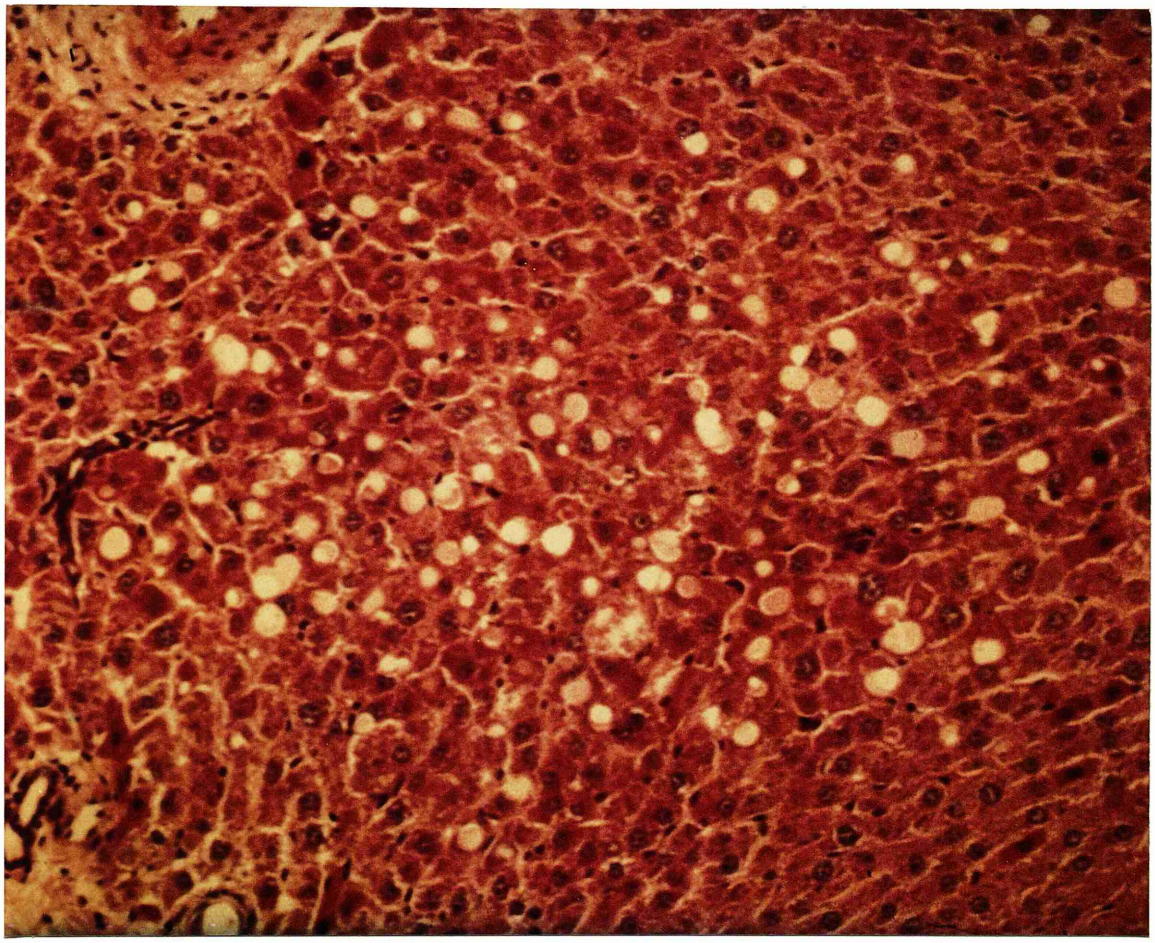

Figure 3.8

Light microscopic appearance of liver 10 weeks after portacaval anastomosis. Marked fatty infiltration.

Haematoxylin and eosin. Magnification X 400.

between peripheral and central nuclear density was no longer observed.

Electron microscopy of the liver

Although a relatively small amount of material was evaluated by this method, several changes were observed in the PCS animals. In a number of cases a variable density of staining was noted, giving the appearance of alternate light and dark cells. This appearance was not seen in PCT or control animals. The most marked change was in the appearance of the mitochondria, which were uniformly rounded in shape, often with a surrounding membrane and with a reduced number of cristae. The cristae often appeared to be in the process of dissolution, and the mitochondria also contained numerous dense osmophilic inclusion bodies. By contrast in the PCT rats the mitochondria were of normal shape with numerous cristae. In the PCS animals the endoplasmic reticulum was scanty, and the glycogen granules had a more lightly flocculated and "open" appearance. These appearances are illustrated in Figures 3.9 and 3.10.

Other organ weights

In the chronic studies carried out in Glasgow, the heart, kidneys, spleen, and testes were also removed and weighed. The only significant difference observed amongst the groups was a reduction in the relative testicular weight after PCS, compared with PCT and control animals (C.J.Ryan, unpublished data: detailed results not included in this Thesis).

Figure 3.9

(Following page)

Electron microscopic appearance of liver 10 weeks after portacaval anastomosis. The mitochondria are rounded and have attenuated cristae.

Magnification X 18,000.

Figure 3.10

(Following page)

Electron microscopic appearance of liver 10 weeks after portacaval transposition. Note several normally shaped mitochondria in contrast to Figure 3.9.

Magnification x 10,000.

Hepatic enzyme changes

(a) Acute studies

Changes in AST were observed at intervals up to 3 days after operation, according to the protocol described in Section 3.2 above. In the initial Glasgow study there were only three animals in each group, with additional groups of control animals (described below). In the comparable UCT study, six animals were used in each group. A complete tabulation of all individual AST values is given in Table 3.6. It can be seen that there was no systematic difference between the two centres, so that these results have been combined for purposes of further analysis. Median values of AST for each time point are shown in Figure 3.11.

The peak median level in PCT rats was 560 u/l (range 427-2400) at 6 hours after operation. In the PCT group, of a total of 53 samples collected during the first 72 hours there were only 9 samples with a value over 500 u/l, and only three with values above 750. In contrast, PCS animals had a peak median AST level of 2,400 u/l (range 520-7245), which occurred 12 hours after operation. In this group, only six of 42 samples during the first 48 hours had values of 500 u/l or less. There was a modest rise in the AST level in control animals, but there was only one value of greater than 500 in the entire series (972 u/l at 24 hours), and this was an isolated high reading in this rat.

Because of the widely and unevenly distributed values, statistical evaluation of these data was performed using

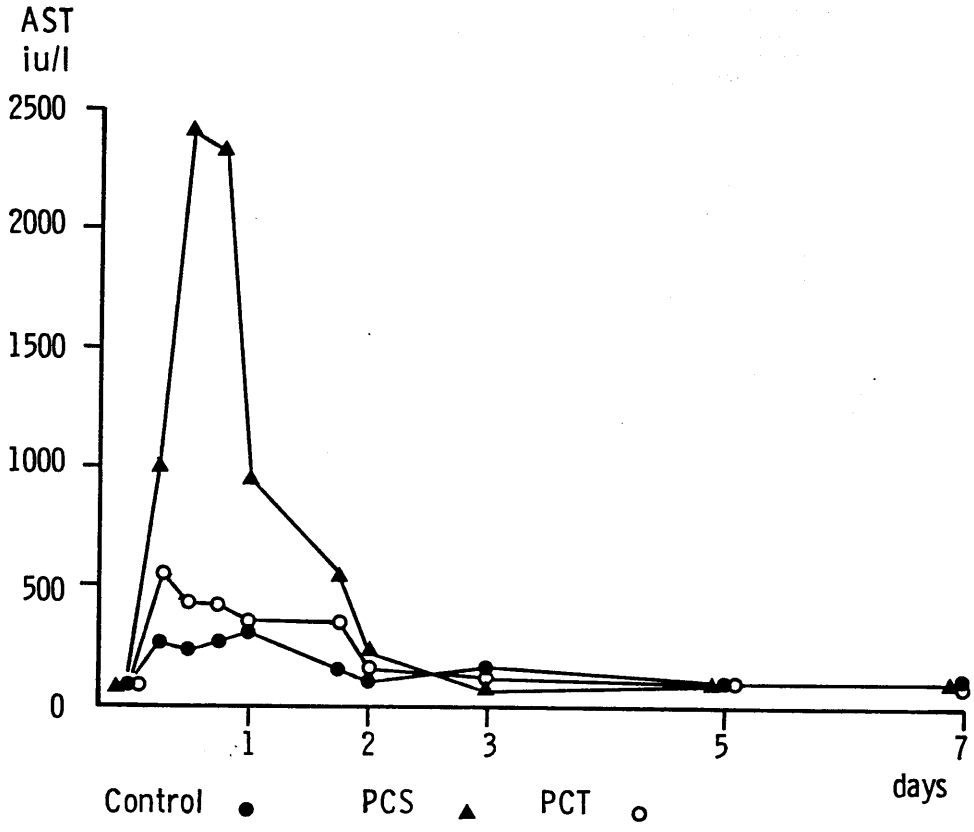

Figure 3.11

Median plasma AST values (i.u./l) from pre-operative values to 7 days after operation (control, PCS or PCT).

TABLE 3.6

AST Levels (iu/l) at 6 hours to 7 days after operation. Both Cape Town (UCT) and Glasgow figures are shown, with combined median values for each operative group. Superscript letters a/b and c/d identify the sub-groups used for blood sampling in the UCT and Glasgow series respectively. Significance levels calculated using Mann-Whitney U statistic.

	HOURS POST-OPERATIVE											
	0	0	6	12	18	24	42	48	66	72	120	168
CONTROL												
UCT	120[a]	70[b]	210[a]	280[b]	210[a]	265[b]	150[a]	90[b]	150[a]	180[b]	—	—
	70	90	225	210	370	250	140	90	130	305	—	—
	100	90	105	190	260	200	150	80	100	80	—	—
	100	165	250	250	310	130	100	130	50	—	—	—
	120	85	300	370	380	240	320	95	260	—	—	—
	110	100	290	180	300	—	110	130	70	—	—	—
Glasgow	91[c]	106[d]	298[c]	269[c]	221[c]	196[c]	—	128[d]	—	245[d]	118[d]	128[d]
	99	119	396	223	211	972	—	141	—	164	96	117
	80	108	406	236	226	204	—	130	—	160	135	103
Median	100		276	245	276	307	162	113	154		116	117
TRANSPOSITION												
UCT	100	70	560	430	425	350	200	125	75	100	—	—
	70	70	2400	460	410	440	200	340	160	120	—	—
	80	75	710	750	1200	370	500	150	150	65	—	—
	95	70	1880	315	490	510	720	270	145	95	—	—
	105	90	—	—	220	500	—	310	120	145	—	—
	70	110	—	—	—	370	—	160	—	60	—	—
Glasgow	101	260	447	320	310	312	—	171	—	291	135	159
	143	165	553	450	421	286	—	141	—	259	86	79
	118	132	427	357	297	414	—	107	—	67	115	107
Median	101		560	430	421	370	350	160	120		115	107
				***		***	*	***				
SHUNT												
UCT	90	115	550	2400	630	960	395	765	110	90	—	—
	100	135	500	710	630	730	315	210	90	100	—	—
	105	95	950	4400	2240	1500	670	330	160	100	—	—
	115	110	1560	550	13400	450	4700	195	560	—	—	—
	120	90	520	1140	1280	690	840	250	85	—	—	—
	155	105	990	520	2600	340	305	150	95	—	—	—
Glasgow	121	186	4251	7245	9783	9586	—	226	—	80	171	112
	117	107	1257	2455	2363	1543	—	146	—	61	114	104
	115	91	2504	4464	3867	2379	—	126	—	132	95	160
Median	114		990	2400	2363	960	533	210	98		114	112
				***	***	***	***	***				
				+++	+++	+++	+++	+++				

*** p vs. control <0.001
* p vs. control <0.05
+++ p vs. PCT <0.001

non-parametric statistics. Using the Mann Whitney U-test, PCS animals differed from controls at 12,18,24,42 and 48 hours ($p < 0.001$), and from PCT animals at 12,18 and 24 hours ($p < 0.001$). PCT animals differed from controls at 12,24 and 48 hours ($p < 0.001$), and also at 42 hours ($p < 0.05$).

In the Glasgow experiments, the studies of acute enzyme changes following portal diversion were extended to include measurement of other enzymes in addition to AST, and additional operative procedures were also undertaken and studied during the same time course. The additional enzymes studied were alanine aminotranferase (ALT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), and creatine kinase (CK). In addition to the rats undergoing PCS, PCT and laparotomy with portal venous clamping as described previously, four further groups of rats were subjected to the following additional procedures:

(i) Laparotomy (LAP): This consisted of laparotomy with mobilization of the liver but without dissection or clamping of vessels.

(ii) Portal vein ligation (PVL): Ligation of the portal venous branch to the anterior lobes of the liver (68% of liver mass), leaving intact portal venous drainage through the posterior lobes.

(iii) Hepatic artery ligation (HAL): The common hepatic artery was mobilized from the portal vein and ligated.

(iv) Partial hepatectomy (PH): This was hepatectomy of the anterior lobes of the liver, according to the technique of

Higgins and Anderson (1931). We have previously shown that in our laboratory this produces a resection of 68.4% of liver mass (± 0.61%, SEm).

The protocol for blood sampling before and after operation was similar to that previously described. Each operation group consisted of 6 animals, divided into two subgroups of 3. The first group was bled at 6,12,18 and 24 hours after operation, and the second at 48,72,120 and 168 hours (7 days).

The individual AST values from 6 hours to 7 days are shown for the additional groups in Table 3.7, with median values taken from Table 3.6 for the original three groups (Control, PCT and PCS) included to facilitate comparison. Median values from the additional groups are also shown in Figure 3.12, which may be compared with Figure 3.11. Full data for all of the enzyme values in these additional groups are included in Appendix B, Table 2.

Because of the small numbers in these groups, statistical evaluation is not appropriate. It can be seen, however, that (as for AST) the highest values of ALT, LDH, and CK occurred in PCS rats, or in rats which had undergone major portal vein ligation (PVL). This emphasizes the importance of portal tract perfusion in these models.

Values for the haematocrit were examined in these acute experiments at 12 and 24 hours. The mean fall in haematocrit was 8.9% (mean difference of 22 paired readings, ± SEm 0.90). There were no significant differences amongst the various groups in the fall in haematocrit.

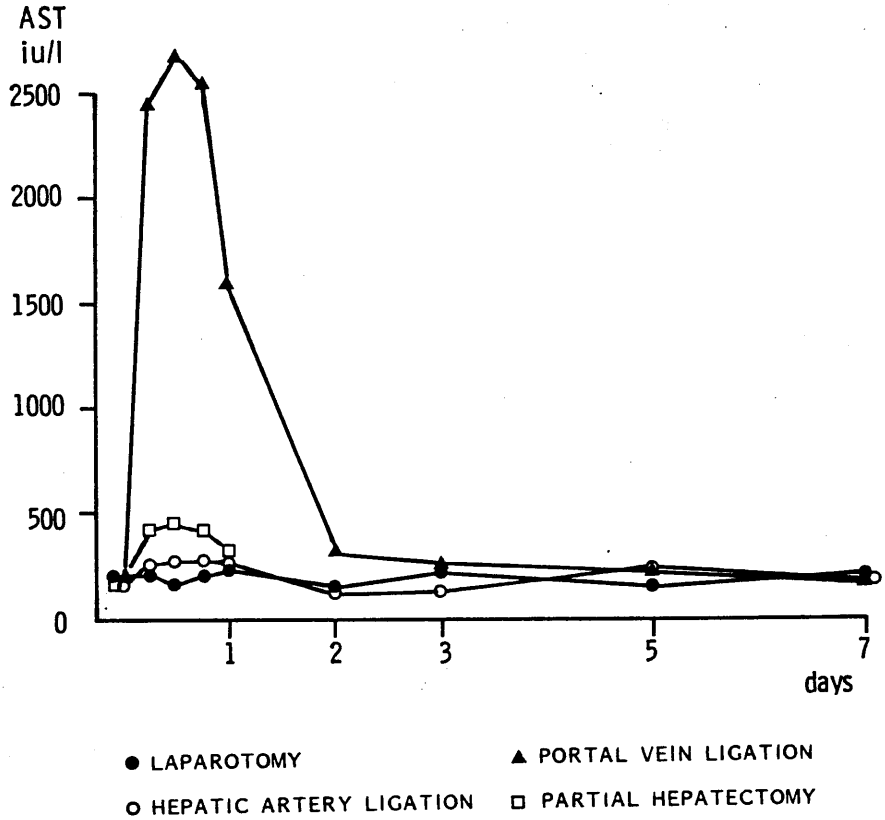

Figure 3.12

Median plasma AST values (i.u./l) from pre-operative values to 7 days after operation in the additional groups used in the Glasgow experiment (laparotomy, portal vein ligation, hepatic artery ligation, and partial hepatectomy).

TABLE 3.7

Serum levels of AST in "acute" studies, from 0 to 168 hours post-operatively. The values for Control, PCT and PCS are median values, taken from Table 3.6. Those for laparotomy alone (LAP), portal vein ligation (PVL), hepatic artery ligation (HAL) and partial hepatectomy (PH) are individual values in groups of three rats. See text for details of operative procedures.

Hours post-op	0	6	12	18	24	42	48	66/72	120	168
CONTROL	100	276	245	276	307	162	113	154	116	117
PCT	101	560	430	421	370	350	160	120	115	107
PCS	114	990	2400	2363	960	533	210	98	114	112
LAP	114	131	78	122	114		131	204	165	164
	175	120	135	148	163		128	175	87	87
	133	130	109	99	193		116	224	84	180
PVL	134	7716	6369	6375	4797		318	277	144	134
	144	2414	2704	2490	1538		361	260	143	110
	131	998	1109	867	655		431	228	128	103
HAL	88	215	209	230	200		118	84	156	132
	94	268	252	236	330		92	90	207	109
	163	215	231	195	172		67	108	112	122
PH	117	310	354	318	223					
	–	394	424	342	310					
	108	315	338	316	212					

(b) Chronic studies

In the Glasgow studies AST, ALT and AP were estimated at weekly intervals up to 5 weeks in control, PCS and PCT rats. In the Cape Town studies only AST was estimated, and the tests were continued for 10 weeks after operation. The results are shown in Figures 3.13 and 3.14. In order to make comparison easier, values in these figures are shown as percentage increase or decrease in each animal from the pre-operative value. The actual values of these enzymes (u/l) are shown in Appendix B, Tables 3-5 for the Glasgow series, and Table 6 for the Cape Town series.

It can be seen from these chronic studies that enzyme levels after PCT did not differ significantly from control values at any time. Enzyme rises following PCS were generally modest, although there were some significant differences from control (at 2 and 3 weeks for AST, at 2,3 and 5 weeks for ALT, and at 2-5 weeks for AP). ALT levels were significantly higher after PCS and after PCT at 1,2,3 and 5 weeks (see Figure 3.13). However, the most striking changes were those seen in the first week following operation, and in particular in the first 72 hours (Figure 3.11), following which enzyme changes were of relatively small magnitude, even in the PCS group. Changes in ALT, LDH, CK and AP in the acute experiment were not as marked as those of AST, although values of ALT at this time were consistently higher in the PCS group than in the control or PCT groups, which showed no significant rise.

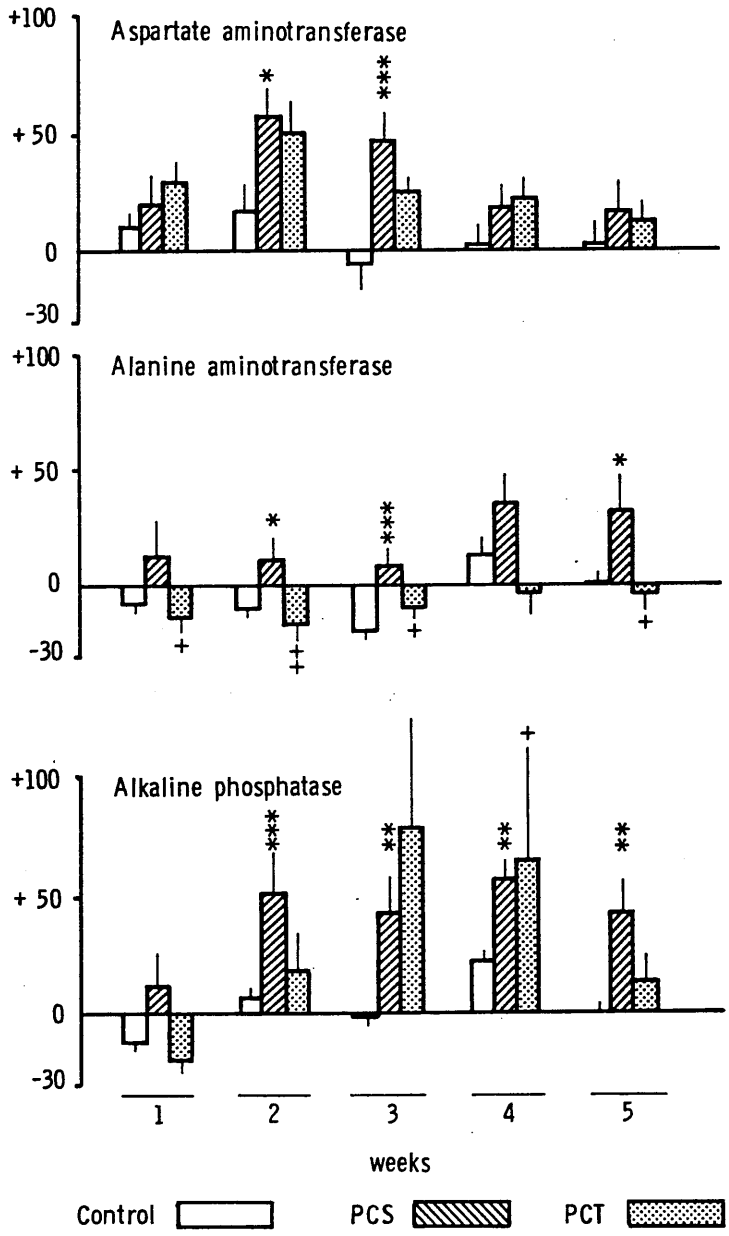

Figure 3.13

Percentage change (pre-operative values considered as zero) in plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase 1 to 5 weeks after operation (control, PCS, PCT). Bars represent means and error bars standard errors of the mean. Statistically significant differences between groups:

PCS vs Control * $p<0.05$ PCT vs Control + $p<0.05$

** $p<0.01$ ++ $p<0.01$

*** $p<0.001$ +++ $p<0.001$

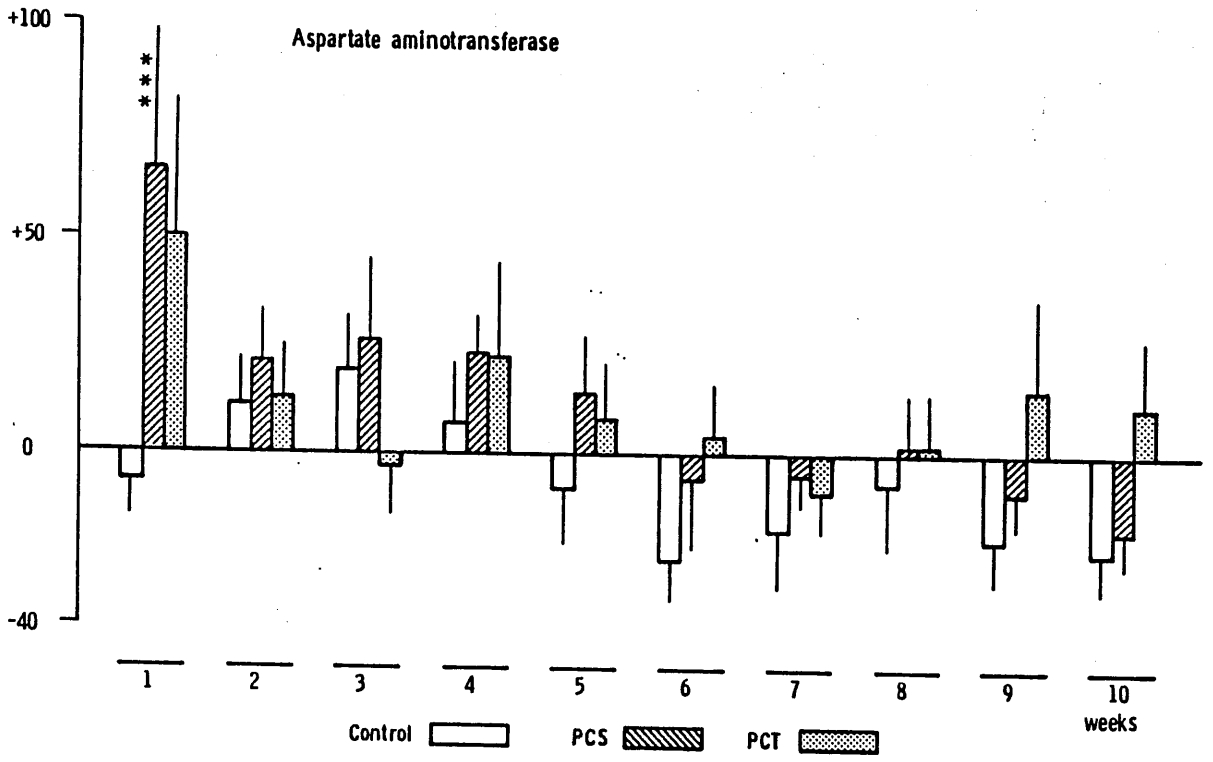

Figure 3.14

Change in plasma AST levels (pre-operative value considered as zero) 1 to 10 weeks after operation (control, PCS, PCT) in ad lib fed rats. Bars represent means, and error bars standard errors of the mean.
PCS vs. Control *** $p<0.001$

Serum protein changes

A radial immunodiffusion assay technique was used for measurement of albumin, IgG and slow $\alpha_1$globulin. Samples from any one rat were always measured on the same assay plate so that inter-plate variation would not affect accurate evaluation of changes in each individual animal, and all protein results are expressed as percentage change from the pre-operative level. The mean values for changes in these three groups from 1-5 weeks are shown in Figures 3.15-3.17, and in summary form in Table 3.8. All values, means and standard errors are tabulated fully in Appendix B, Tables 7-9.

Serum albumin levels fell in all three groups, although in the control group the fall was transient. The percentage fall in albumin was significant from the first week onwards in PCS animals, but only reached a statistically significant level at weeks 4 and 5 following PCT (see Table 3.8). It was not possible to demonstrate any statistically significant difference at individual time points between PCS and PCT animals. However, the data for the first three weeks after operation may be pooled for each of these two groups, giving for each group the mean of 33 observations in 11 animals, since there are no significant trends in level or differences in variance within each group during this period (Campbell 1974). This results in a mean percentage fall in serum albumin of 20% ($\pm$ 4 SEm) for the PCT group and 30% ($\pm$ 3 SEm) for the PCS group, a difference which is statistically significant ($p < 0.05$, Student's unpaired t).

Slow α_1- globulin rose post-operatively in all three

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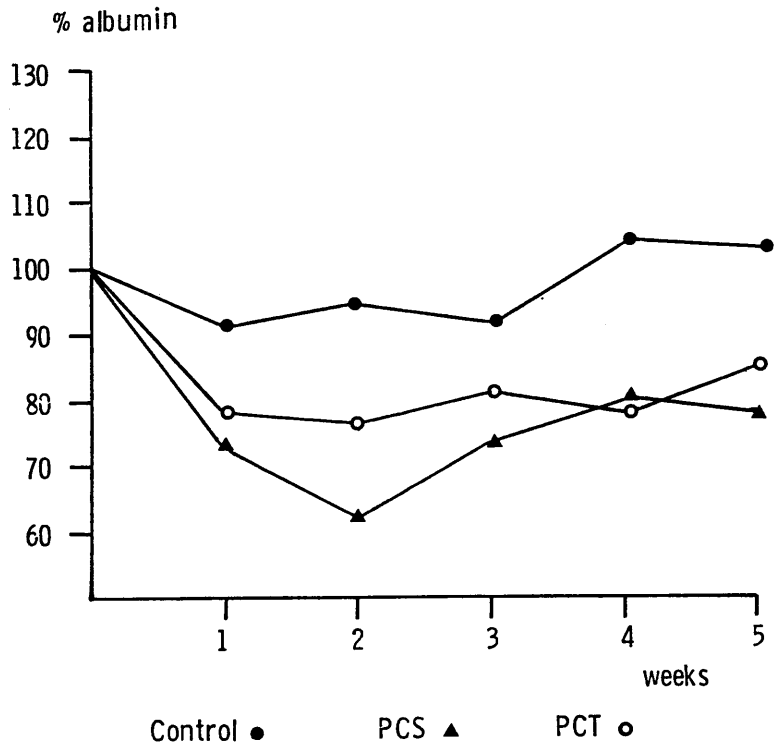


Figure 3.15

Plasma albumin levels as percentage of pre-operative level, 1 to 5 weeks after operation in ad lib fed rats. Each value is the mean for one group. Error markings are omitted for clarity. Values are given in Appendix B, Table 7.

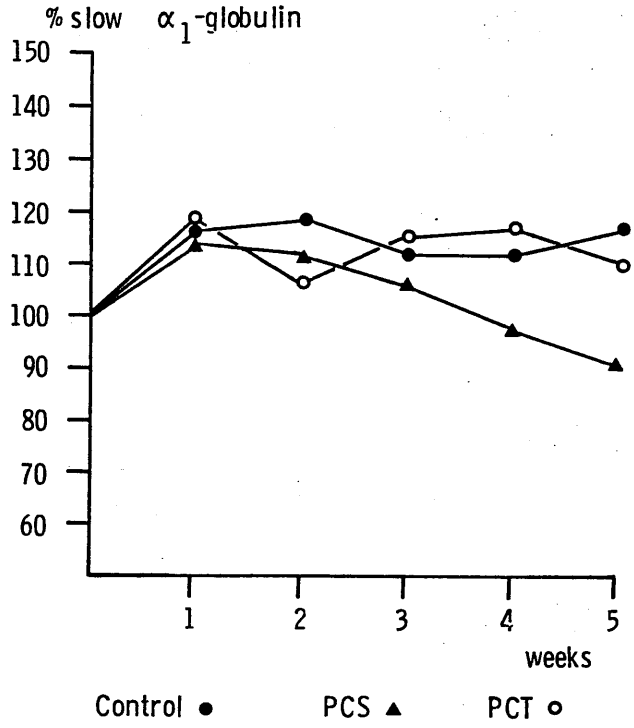


Figure 3.16

Plasma slow α_1 -globulin levels as percentage of pre-operative level, 1 to 5 weeks after operation in ad lib fed rats.
Each value is the mean for one group. Error markings are omitted for clarity. Values are given in Appendix B, Table 8.

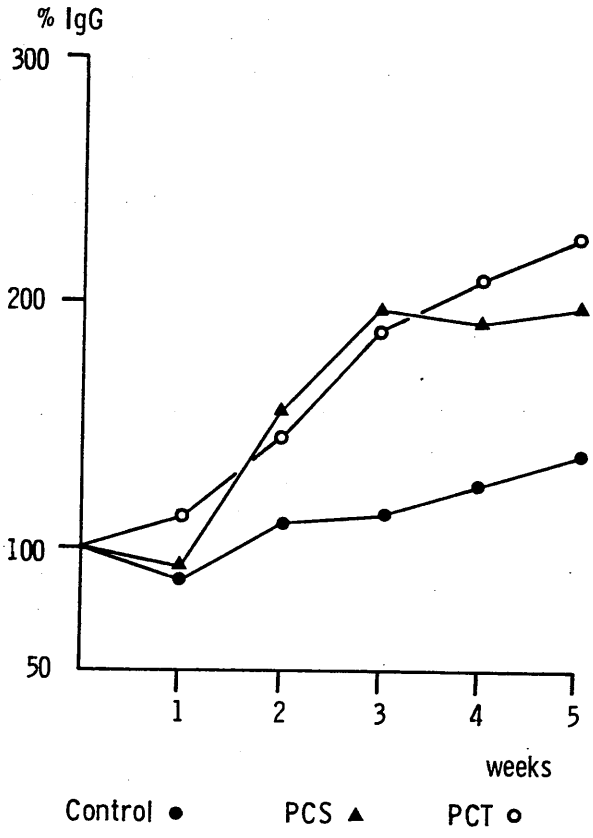


Figure 3.17

Plasma IgG levels as percentage of pre-operative level, 1 to 5 weeks after operation in ad lib fed rats. Each value is the mean of one group. Error markings are omitted for clarity. Values are given in Appendix B, Table 9.

TABLE 3.8

Protein levels (as % of pre-operative level) 1-5 weeks after operation (mean ± 1 SEm)

	W E E K S				
	1	2	3	4	5
ALBUMIN					
Control (18-19)	92 ± 5	95 ± 6	93 ± 5	105 ± 6	104 ± 5
PCT (11)	79 ± 7	77 ± 8	82 ± 6	*80 ± 4	*86 ± 6
PCS (10-11)	*74 ± 5	***63 ± 6	*74 ± 6	*81 ± 8	**79 ± 6
SLOW α_1-GLOBULIN					
Control (18-19)	117 ± 4	119 ± 4	113 ± 3	113 ± 4	118 ± 5
PCT (11)	118 ± 5	107 ± 4	116 ± 3	118 ± 4	112 ± 5
PCS (10-11)	115 ± 4	112 ± 3	107 ± 3	++ **99 ± 4	++ **93 ± 5
IgG					
Control (18-19)	88 ± 7	111 ± 7	115 ± 8	126 ± 7	139 ± 11
PCT (11)	113 ± 11	*146 ± 16	***188 ± 17	***208 ± 20	***224 ± 20
PCS (10-11)	93 ± 9	**156 ± 12	***198 ± 12	**192 ± 18	**197 ± 19

STATISTICS: Student's unpaired t-test, significantly different from *control
+PCT

Levels of significance:

*	+	$p < 0.05$
**	++	$p < 0.01$
***	+++	$p < 0.005$

groups. This new level was sustained throughout the period of study in control and PCT animals, but began to fall again from the third week onward following PCS. At the fourth and fifth week the S α_1-globulin level had fallen below the starting values in the PCS animals, and at these time points there was a statistically significant difference between PCS and both control and PCT levels.

IgG rose by 39% at the fifth post-operative week in control animals, but in the PCS and PCT groups the level rose much more rapidly, increasing by 97% and 124% respectively by the end of the study. There was at no time a significant difference between the PCS and PCT levels, but both groups differed significantly from control animals at all points from the second week onwards.

A separate study of serum IgA levels after PCS and PCT was also performed in collaboration with Dr. HC Thomas (Royal Free Hospital, London) and with Dr. JP Vaerman (University of Louvaine, Brussels). These experiments, using 7 PCS, 6 PCT, and 5 control animals, demonstrated a massive (15 to 23-fold) increase in serum IgA levels following PCS, with no corresponding change following PCT or control operation. IgM levels also showed a moderate (1.7 to 2.2-fold) increase following PCS, but none after PCT. In these studies the changes in IgG were less marked than those described in the previous experiment. This is thought to be due to different specificity of the antisera to IgG subclasses between the two experiments.

Since the IgA work is related only obliquely to the studies under discussion, these experiments will not be considered

further here. The relevant publication (Vaerman et al, 1981) is included in Appendix C.

4.2 METABOLIC STUDIES (Glasgow)

In this controlled feeding study, 11 animals were used (4 PCS, 4 PCT, 3 controls). Full results from a representative member of each group are shown in Figures 3.18-3.20. All of the results for immediate post-operative weight loss and total weight gain by the end of the six-week experiment are shown in Table 3.9, with the relevant summary tables for one-way analysis of variance. The figures illustrate the fact that following a period of weight loss lasting up to one week, the animals regained a normal growth pattern, with a similar rate of growth in all three groups. The mean maximum post-operative weight loss was not significantly different amongst the groups. The mean maximum weight loss in PCS rats was 15.3% (± 4.7 SEm): this is not significantly different from that observed in the studies described above in which animals were fed *ad lib* and housed four to a cage. However, PCT rats lost a mean of 10.9% (± 1.3) of their body weight, in contrast to the *ad lib* fed animals in which the mean maximum weight loss was 4.5% (± 1.7%), a significant difference ($p < 0.01$, Student's unpaired t). The mean maximum weight loss in control animals (9.6%) was also greater than that in the previous study (4.1%), although the range was wide in the present metabolic study and this was not a statistically significant difference.

At the conclusion of the experiment (6 weeks), there was a mean weight gain of 24.8% in PCS rats, with corresponding gains of 33.1% and 33.5% in PCT and control animals respectively. Thus up to 6 weeks there were no significant differences amongst the growth characteristics of these groups. Again, this is in marked

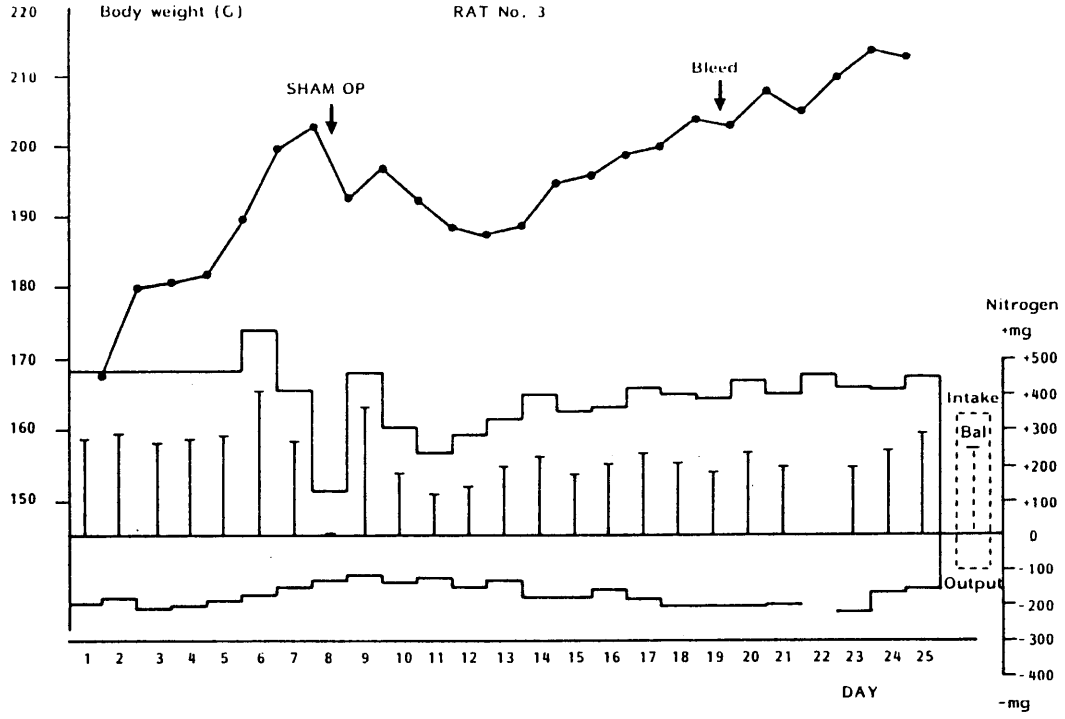
growth characteristics of these groups. Again, this is in marked contrast to the ad lib feeding studies, in which the body weight at 6 weeks relative to the pre-operative weight was 92.4% (± 3.9%) for PCS animals, 140.6% (± 4.2%) for PCT animals, and 146.1% (± 3.2%) for control animals (all ± 1 SEm). These differences are discussed below.

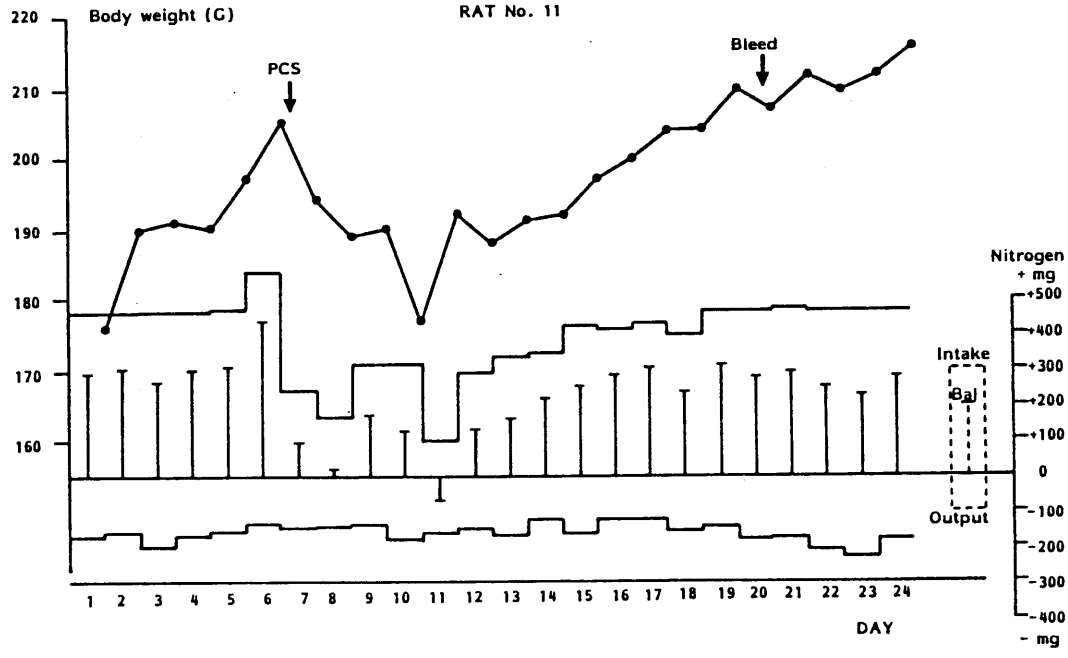
The daily food nitrogen intake and urinary nitrogen output are also shown in Figures 3.18-3.20. Only a transient negative nitrogen balance in the post-operative period was observed in these studies, which of course do not take account of faecal nitrogen losses. On the present measurements, there was a net positive nitrogen balance of between 100 and 400 mg per day throughout the studies.

4.3 PAIR FEEDING EXPERIMENTS (Cape Town)

The body weight changes seen during this study are shown in Figure 3.21 which should be compared with Figure 3.1 showing weight changes in animals fed ad lib for a similar time period. In this experiment there was an initial period of weight loss lasting up to 3 weeks, during which control and PCT animals lost a comparable amount of body weight (maximum 8-13%). Following this, however, there was a period of relatively slow growth, with no significant differences amongst the groups. Full results are tabulated in Appendix B, Table 10.

The liver weight at sacrifice in these animals is shown in Figure 3.22, which should be compared with Figure 3.2, which contains the comparable data for ad lib fed animals. Table 3.10 compares the data for wet and dry relative liver weight in the ad





Figures 3.18 and 3.19

Examples of results in two individual rats (Control and PCS) in the Glasgow metabolic experiment. Note that the PCS rat (No 11, lower curve) was operated upon one day before the Control rat (No 3, upper curve), which was thereafter given on each subsequent day the same quantity of food consumed by the PCS rat the day before. The results for the the PCT rat operated on the same day and also pair fed against rat No 11 are shown in Figure 3.20, where a full explanation of the axes is also given.

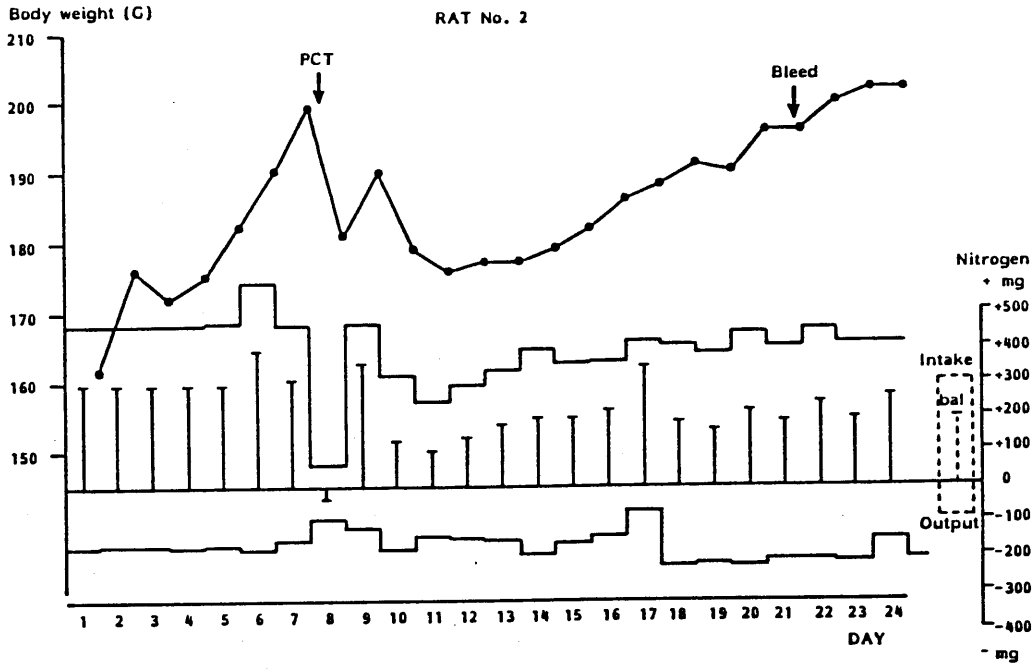


Figure 3.20

Results for Rat No 2 (PCT) in the Glasgow metabolic pair-feeding study. The layout is identical to the previous two Figures (3.18,3.19), which represent the results of rats in the same grouping as shown here.

The continuous line shows the daily body weight (G, left-hand axis). The blocks above and below the horizontal zero line show the daily nitrogen content (mg, right-hand axis) of consumed food (above the line) and urinary nitrogen loss (below the line). The vertical bar within each day's block represents the difference between these two values, i.e., the daily positive or negative measured nitrogen balance.

Note that after an equilibration period of 6 days in the metabolic cages, the PCS rat first underwent surgery, and the following day both the Control and PCT procedures were carried out. Following the operation food consumption fell for several days in the PCS rat, and the quantity of food consumed by that animal was made available to each of the other two in the group on subsequent days. The point at which the animal was anaesthetized and blood sampled is shown on each graph.

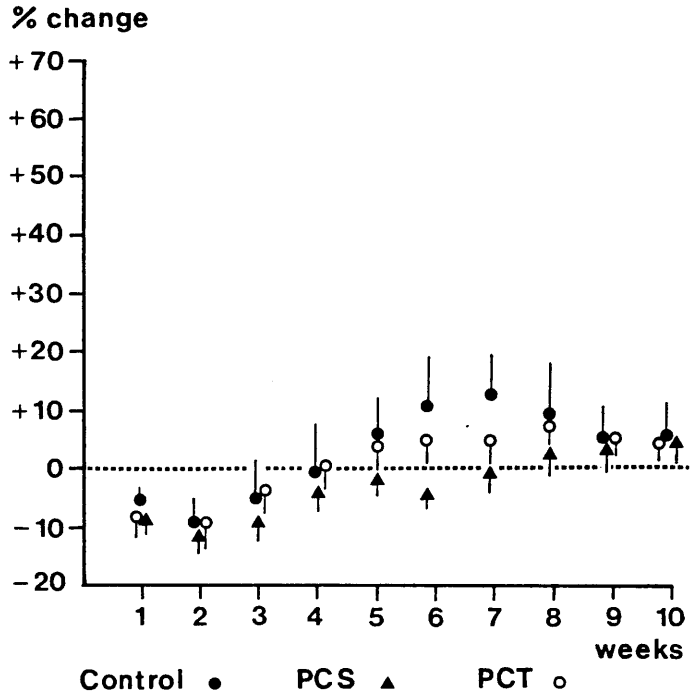


Figure 3.21

Percentage change from pre-operative body weight in pair-fed rats 1 to 10 weeks after operation.
(Compare with Figure 3.1)
Each point is the mean of one group.
Error bars show standard errors of the mean.

TABLE 3.9

Changes in body weight in rats housed in metabolic cages, with pair-feeding of control and PCT rats against PCS (Glasgow study).

	Cage No	Pre-op BW	6 wks	Gain	Min. BW	Max. wt.loss
Control	3	203	279	37.4%	188	7.4%
	5	194	271	39.6%	183	5.7%
	9	199	246	23.6%	168	15.6%
	Mean			33.5%		9.6%
	SEm			(± 5.0%)		(± 3.1%)
PCT	1	186	246	32.3%	160	14.4%
	2	198	280	41.4%	176	11.1%
	4	194	277	42.8%	174	10.3%
	8	215	249	15.8%	198	7.9%
	Mean			33.1%		10.9%
	SEm			(± 6.2%)		(± 1.3%)
PCS	6	197	285	44.7%	183	7.1%
	7	183	217	18.6%	170	7.1%
	10	185	206	11.4%	144	22.2%
	11	195	243	24.6%	167	24.6%
	Mean			24.8%		15.3%
	SEm			(± 7.2%)		(± 4.7%)

Summary ANOVAR tables for above data

1). For weight gain at 6 weeks

Source of variation	Sum of squares	Deg of freedom	Mean square	Variance ratio (F)
Between groups	208.95	2	104.475	0.687
Residual	1227.39	8	153.42	

2). For maximum weight loss

Source of variation	Sum of squares	Deg of freedom	Mean square	Variance ratio (F)
Between groups	64.46	2	32.23	0.745
Residual	346.27	8	43.28	

TABLE 3.10

Relative liver weight (% of body weight at sacrifice) 10 weeks after operation in animals fed ad lib and pair-fed. (The data are re-arranged from Table 3.2). Significant differences are marked between the values in the two experiments.

	n	Ad lib fed		Pair-fed	n
Wet weight					
Control	(14)	3.24 ± 0.08	+++	2.55 ± 0.06	(3)
PCT	(15)	2.77 ± 0.09	++	2.47 ± 0.04	(6)
PCS	(14)	2.07 ± 0.10	+	1.83 ± 0.05	(6)
Dry weight					
Control	(5)	1.10 ± 0.06	+++	0.72 ± 0.03	(3)
PCT	(8)	0.94 ± 0.04	+++	0.74 ± 0.01	(6)
PCS	(6)	0.67 ± 0.05		0.58 ± 0.01	(6)

Ad lib vs pair-fed: $p < 0.05$ +
$p < 0.01$ ++
$p < 0.005$ +++

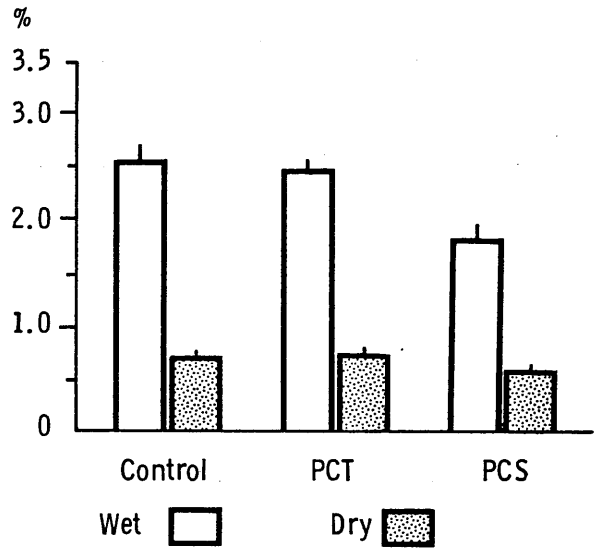


Figure 3.22

Relative wet and dry liver weights (liver weight as percentage of body weight at sacrifice) 10 weeks after operation in pair-fed rats.
(Compare with Figure 3.2)
Each bar shows the mean relative liver weight for rats of one group. Error bars show standard errors of the mean.

Table 3.2). It can be seen that there is a highly significant reduction of both wet and dry liver mass in the control and PCT animals resulting from pair feeding, and also a smaller reduction in liver mass in the PCS group (statistically significant for wet weight but not for dry weight). Nevertheless, the overall relationship amongst the groups remains undisturbed, with a significant reduction in relative liver weight (both wet and dry) between the PCS and both PCT and control groups ($p < 0.001$). Although in the ad lib fed animals there is a statistically significant reduction in liver weight in the PCT rats compared to control, the small difference observed in the pair fed animals does not reach statistical significance.

Further biochemical and histological results obtained from this pair feeding experiment are of relevance to other parts of these studies. It is more appropriate, however, to consider these changes in their immediate context, so that presentation and discussion of additional results in pair fed animals is deferred until later sections of this Thesis (Chapter IV Section 2 and Chapter V Section 4).

4.4 ROSE BENGAL CLEARANCE MEASUREMENTS

Methods

These studies were carried out in Cape Town, on Long Evans rats housed and fed ad lib according to the previously described protocols, and examined 10 weeks after operation. At the time of clearance measurement the weight range of these animals was 358-625 g.

The object of these studies was to measure and analyse the kinetics of the disappearance of ^{131}I-labelled Rose Bengal from the circulation as a measure of hepatic perfusion and hepatocellular clearance function. The isotope was obtained from the Radiochemical Centre, Amersham, England, and was diluted in saline before use to give a Rose Bengal concentration of 0.03 mg/ml, and a specific activity of 20 μCi/ml.

Anaesthesia was induced and maintained with open ether. The rat was fixed supine on a board with the tail hanging downwards over the edge of the bench. Blood samples were obtained by cleanly cutting off the last 2-3 mm of the tip of the tail, and applying a microhaematocrit capillary tube ("Red Tip", Sherwood Medical Industries Inc., St. Louis), to the drop of blood which formed at the cut surface. Bleeding was then stopped by gentle pressure, and could be restarted by wiping the cut surface with a gauze swab. The first drop of blood obtained on restarting the bleeding process in this way for each sample was wiped away and discarded to ensure sampling of freely circulating blood.

After removing an initial baseline sample, 1 ml of the isotope was injected slowly into the dorsal vein of the penis using a 25 gauge hypodermic needle. The injection lasted up to 20 seconds, following which timing was commenced and pressure was applied to the vein for haemostasis. Further capillary tube samples were then taken from the tail tip at one minute intervals until 20 minutes after injection, and then at one or two minute intervals until 30 minutes.

Each filled capillary tube was rested in an empty

scintillation counter cuvette, and using a syringe of water with a length of silicone tubing attached the traces of blood remaining in each capillary tube were carefully washed into the cuvette. The volume of water used was approximately 1 ml. (This volume is not critical, and tests performed using volumes between 1 and 2 ml for the washing process demonstrated that there is no significant effect on the counts obtained. It is important, however, to ensure that the haematocrit tube is placed accurately in the bottom of the cuvette, since soiling with blood higher up the walls of the cuvette may result in inaccurate counting.)

Cuvettes were then counted for one minute in an LKB liquid scintillation counter. After correcting for background counts, clearance curves were constructed for initial inspection either by manual plotting on semi-logarithmic paper, or by computerised curve construction using a Tektronix desk-top microcomputer with graphics facility. Following this, more detailed mathematical analysis of the curves was performed using the P3R multiple linear regression programme of the BMDP package on a Univac 1100 computer. The sample counts were logarithmically transformed, and weighting was applied using the inverse of the log counts as a weighting factor.

Rose Bengal clearance results

This simplified technique, which was developed for measurement of blood clearance with a minimum of specialized equipment, relies on the reproducibility of the sampling volume obtained using microhaematocrit tubes. A number of commercially available capillary tube types were examined, and the Red Tip

tube from Sherwood Medical Industries was chosen because of the replicability of the sampling volume. These tubes have a nominal length of 75 mm, outside wall diameter of 1.1-1.2 mm, and wall thickness of 0.2 ± 0.02 mm. Replicability was tested several times by counting 20 tubes filled from a reservoir of diluted radio-labelled Rose Bengal, and washed into cuvettes exactly as described for the clearance test in rats. The mean count obtained on this test was 41,581 ± 233 (SEm). This is a 2.5% coefficient of variation arising from variations in tube dimensions, operator error, and counting scatter. This overall level of random variation is considered to be an acceptable limit of accuracy for the technique as a whole.

Results were available for analysis from 4 control, 6 PCT and 7 PCS animals. A typical clearance curve from one animal in each group is shown in Figures 3.23-3.25. The theoretical three-compartment model which may be used to describe clearance of material extracted by the hepatocytes and excreted into the bile is illustrated diagramatically in Figure 3.26. Regression lines were fitted in accordance with this model using the BMD P3R programme, and these regression lines have been drawn in Figures 3.23-3.25. Table 3.11 shows the computed values for a and b (the y intercepts) and k_1 and k_2 (the slopes of the two phases of the regression) in all of the animals studied. No significant differences were found between the values of $-k_2$ in the PCS, PCT, and control animals. The values of $-k_1$ did not differ significantly between PCT and control animals, but the value of $-k_1$ for PCS animals differed significantly both from control and from PCT ($p < 0.0005$, Student's t-test).

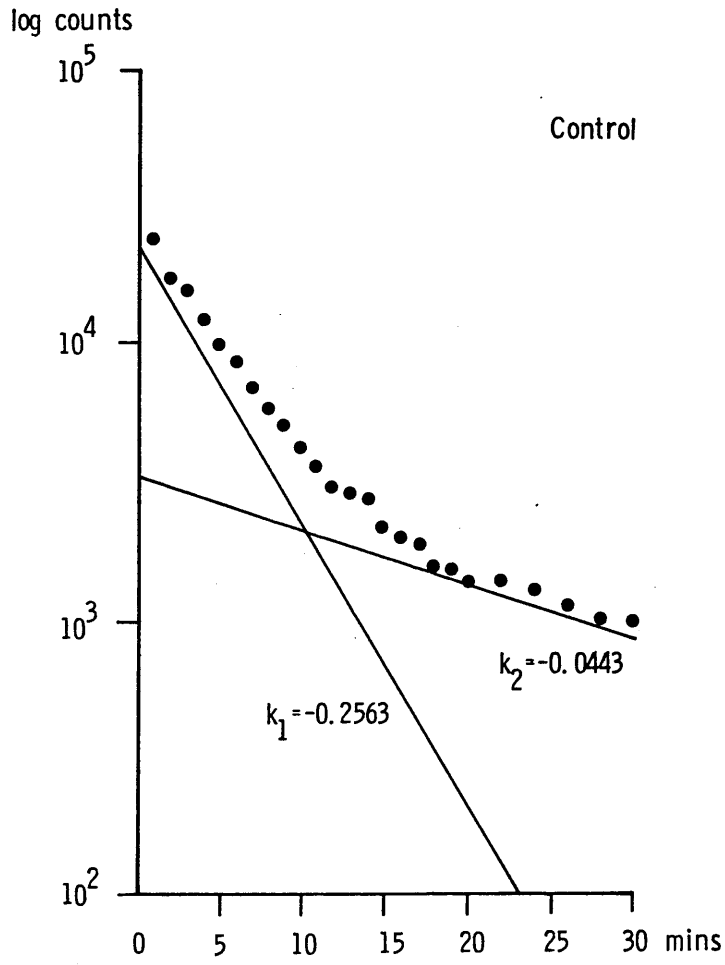


Figure 3.23

Radio-labelled Rose Bengal clearance curve 10 weeks after operation in a single control rat.
Each point shown represents the log of counts/minute in a blood sample at the appropriate time point. The solid lines are the computer-fitted regression lines for bi-exponential clearance kinetics, and the derived values of k for the two components are marked. The method of calculation is described in the text, and values of k_1 and k_2 are shown in full in Table 3.11.

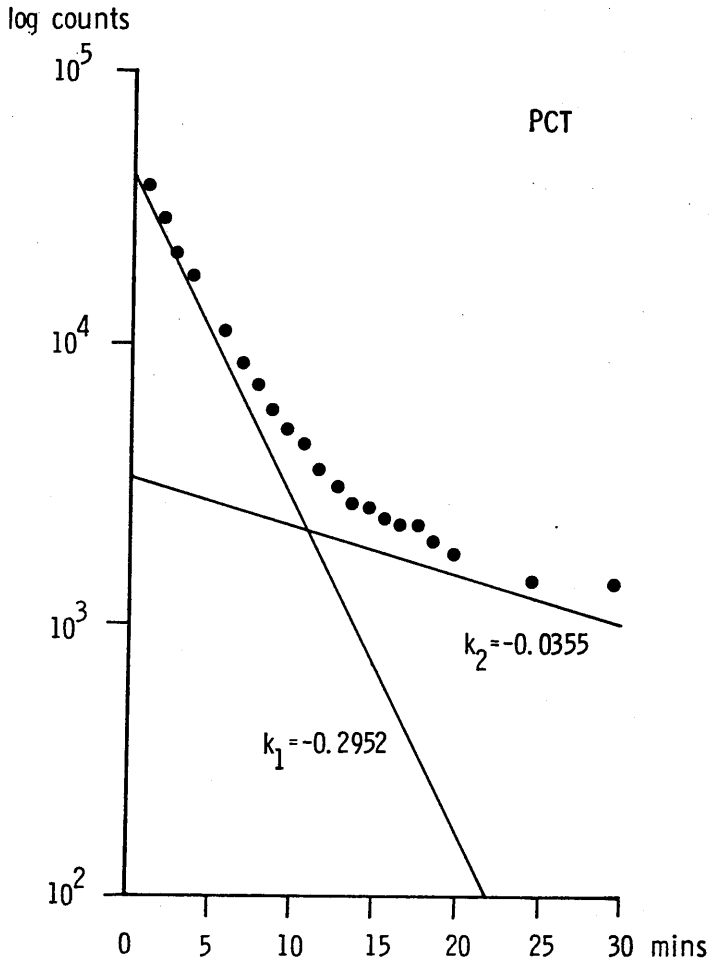


Figure 3.24

Radio-labelled Rose Bengal clearance curve 10 weeks after operation in a single PCT rat.
Each point shown represents the log of counts/minute in a blood sample at the appropriate time point. The solid lines are the computer-fitted regression lines for bi-exponential clearance kinetics, and the derived values of k for the two components are marked. The method of calculation is described in the text, and values of k_1 and k_2 are shown in full in Table 3.11.

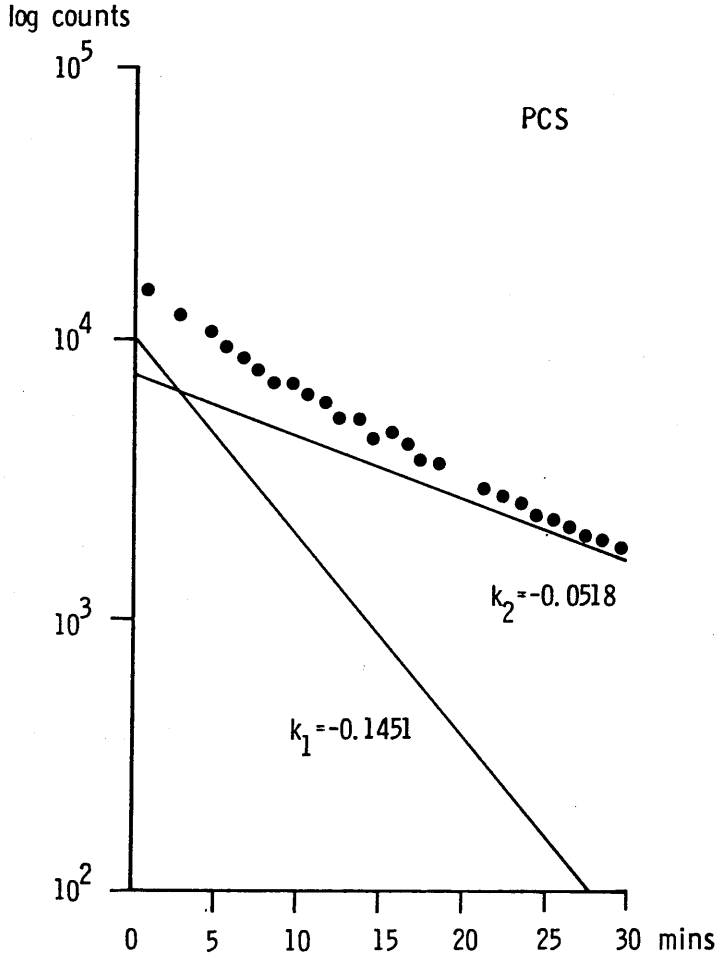


Figure 3.25

Radio-labelled Rose Bengal clearance curve 10 weeks after operation in a single PCS rat.
Each point shown represents the log of counts/minute in a blood sample at the appropriate time point. The solid lines are the computer-fitted regression lines for bi-exponential clearance kinetics, and the derived values of k for the two components are marked. The method of calculation is described in the text, and values of k_1 and k_2 are shown in full in Table 3.11.

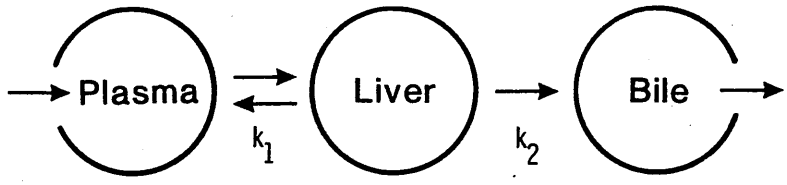


Figure 3.26

Schematic diagram of the three-compartment model of hepatic uptake, clearance and excretion of Rose Bengal (see text).

TABLE 3.11

Computed parameters of Rose Bengal clearance curves (see text)

	A	B	$-K_1$	$-K_2$	
Control	14856	4959	0.3288	0.0986	
	15344	1436	0.3641	0.0450	
	48262	3342	0.2952	0.0355	
	54623	5161	0.2486	0.0499	
			0.3091	0.0573	mean
			± 0.0492	± 0.0282	SD
PCT	51976	3166	0.2472	0.0129	
	28024	1795	0.2774	0.0229	
	47880	1811	0.2660	0.0046	
	42322	4351	0.3378	0.0363	
	26413	3397	0.2563	0.0443	
	14457	3290	0.2405	0.0643	
			0.2709	0.0309	mean
			± 0.0353	± 0.0219	SD
PCS	15814	3055	0.1068	0.0463	
	13724	4963	0.1504	0.0497	
	24412	4620	0.1586	0.0436	
	15720	3963	0.1192	0.0454	
	15718	1841	0.0718	0.0121	
	10254	8587	0.1451	0.0518	
	16102	4370	0.1150	0.0248	
			0.1239*	0.0391	mean
			± 0.0302	± 0.0148	SD

*p vs. Control or PCT < 0.0005 (Student's t-test).

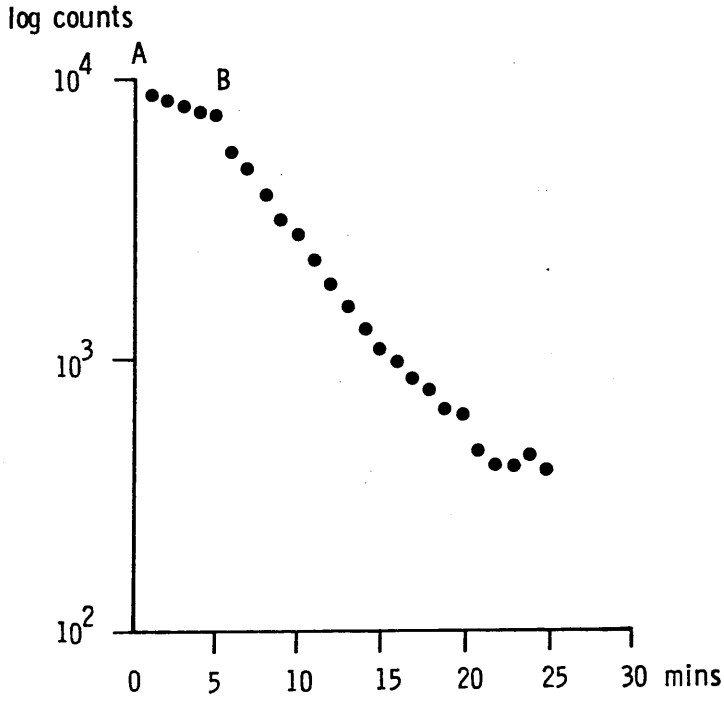


Figure 3.27

Clearance of Rose Bengal in a single normal rat. At point A, after intravenous injection of Rose Bengal, the vascular inflow to the liver was occluded, and clearance at this time is extremely slow. The occlusion was removed at point B, following which more rapid clearance can be seen.
(Axes are exactly as in Figures 3.23-3.25).

Although it is well established that there is little extrahepatic clearance of Rose Bengal, one experiment was conducted to confirm this for the present situation. Figure 3.27 shows the effect of clamping of the hepatic artery and portal vein immediately after injection of the isotope (point A), with release of the clamp five minutes later (point B). There is almost no clearance during the phase of circulatory arrest to the liver, while prompt clearance commences again on releasing the clamps.

4.5 LIVER REGENERATION STUDIES

The model of PCT was first introduced by Child and his colleagues (1953) for the investigation of regenerative hyperplasia of the liver. The model has the major advantage in this situation of allowing the study of liver size and function following partial hepatectomy in the presence of adequate hepatic blood flow, but without direct perfusion of the liver by blood flowing from the intestines and pancreas, the putative source of "portal hepatotrophic factors" (Starzl, Francavilla et al, 1973). While the work carried out in this laboratory on the use of PCT in investigation of liver atrophy, hypertrophy, and regenerative hyperplasia is strictly beyond the scope of this Thesis, I participated in the work during the time span of the present Thesis, and many of its findings are relevant. The paper (Guest et al, 1977) is therefore included in Appendix C, and the important findings in relation to the present investigations are summarized here:

(a) The small but significant reduction in liver size which follows PCT was confirmed in this paper, in which groups of

animals were sacrificed at three and six weeks after PCT.

(b) Partial (68%) hepatectomy carried out three weeks after PCT is followed by restoration of liver mass, with the same time course as that seen in control animals.

(c) The mass which the liver achieves three weeks after partial hepatectomy (6 weeks after PCT) is the same as that reached by the concomitant process of liver atrophy in a period of six weeks in animals subjected to PCT alone (see Figure in Guest et al, 1977 in Appendix C).

(d) The time course and magnitude of the cellular process of regenerative hyperplasia following PCT, as assessed by hepatic DNA activity ratio, is also unimpaired in comparison with control animals.

(e) These results support the general concept that liver atrophy seen after portal diversion is a consequence of loss of trophic substances in the portal blood rather than a decrease in absolute liver blood flow. However, the absence of such factors neither delays the initiation nor impairs the time course of regenerative hyperplasia following partial hepatectomy.

This important study highlights the paradox that the processes of liver atrophy and of regenerative hyperplasia may occur simultaneously in the same animal.

4.6 OVARIAN SPLENIC IMPLANT STUDIES

Ovaries autotransplanted into the spleens of spayed female rats form hyperplastic luteal cell masses (Biskind and Biskind, 1944), due to raised levels of plasma FSH and LH. It has been shown that PCS can prevent this rise, and also arrest the growth and induce atrophy in established implants (Seager et al, 1974). This effect was presumed to be due to the return of portal blood, and therefore of ovarian hormones, into the systemic circulation, restoring the normal physiological feedback between the ovaries and the hypothalamo-pituitary axis. However, in common with other phenomena relating to PCS, the alternative explanation of failure of inactivation of hormones due to progressive liver atrophy and dysfunction must also be entertained. For this reason a series of intrasplenic ovarian implants was established followed 26-38 weeks later by laparotomy with either PCS, PCT or no further procedure. At sacrifice 20 weeks after the second operation, atrophy and histological degeneration of the hyperplastic luteal cell masses was found, and the degree of this change was comparable between PCS and PCT. Although hormone levels were not measured, it seems likely therefore that the changes seen may be accounted for by portal diversion resulting in restoration of the ovarian-pituitary hormonal control mechanism.

Further details of this work are not included in this Thesis, but a manuscript of the work (without photomicrographs) is included for reference in Appendix C.

5. ESTABLISHMENT OF PCT MODEL - CONCLUSIONS

The proposal of PCT as a model of portal diversion with normal liver function clearly represents a degree of compromise. All the results described above show differences between PCS and PCT which we may attribute to maintenance of hepatic perfusion with non-portal blood in the PCT animal. Nevertheless, it must be accepted that the PCT rat does not have a "normal" liver, and attempts to achieve this by other means, such as portal stump arterialization, have achieved similar results (Castaing, Franco et al, 1982). Thus it is appropriate here to summarize the evidence regarding the differences between PCT and PCS.

5.1 Body weight changes

The cause of weight loss following PCS is incompletely established. Our studies and those of others (Assal et al, 1971; Keraan et al, 1974) have shown that there is a marked decrease of food intake, though the reason for the anorexia is not known. We have consistently observed that PCS rats housed in individual cages thrive better than those housed four to a cage, conditions to which normal or PCT rats are not sensitive. Hence the degree of post-operative weight loss in our pair-fed animals, individually housed in metabolic cages, was less than that in the other experiments. The small metabolic study reported here showed normal positive nitrogen balance in these animals: though somewhat surprising, this result is in keeping with the observations of other workers of normal nitrogen balance and normal intestinal absorption of proteins, carbohydrates and

lipids following PCS (Assal et al, 1971; Fisher et al, 1968). Our initial observation that younger rats tolerated PCT better than older ones is also in keeping with previous observations following PCS (Kyu and Cavanagh, 1970), though we have not attempted to elucidate this finding further.

5.2 Liver atrophy

Our results confirm the well-established observation of marked liver atrophy following PCS in the rat. The relative liver mass falls by almost one third at 10-12 weeks after operation. That this is a true loss of liver substance and not merely a difference of perfusion is demonstrated both by the reduction in dry liver mass and by the histological studies which demonstrate marked nuclear crowding. A degree of nuclear crowding would also be consistent with sinusoidal collapse following PCS, but more elaborate studies using tracing techniques have reached the same conclusion as ours (Starzl, Francavilla et al, 1973).

However, it is important to note that PCT does not maintain a completely normal liver mass, despite maintenance of a quantitatively normal total hepatic perfusion (the evidence for which is discussed below). A small but significant reduction in liver mass is demonstrated in these studies, and we have shown elsewhere that this is present as early as three weeks and is slowly progressive (Guest et al, 1977). These findings lend support to the importance of specific "hepatotrophic" factors in portal venous blood for the maintenance of normal liver mass. The origin of such substances has been extensively investigated

in recent years, and they are now widely accepted as being hormonal in nature (Starzl, Francavilla et al, 1973). The evidence that the important hormone is insulin is less clear, although Starzl has shown that selective lobar intraportal insulin infusion in dogs greatly reduces the hepatocyte atrophy produced by portacaval shunting (Starzl et al, 1975). Other workers have shown the importance of insulin in maintaining hepatocyte mitochondrial metabolism (Ozawa et al, 1974). It is important to keep clear the complex distinction between factors which maintain the balance between hepatocyte atrophy and growth, and those which initiate or control the process of "regenerative" hyperplasia following a variety of hepatic injuries including partial resection. The failure to make this distinction led to the long-standing Rous-Mann controversy over the relative importance of flow and of trophic substances (Rous and Larimore, 1920; Mann, 1940). Weinbren in 1955 demonstrated that active mitotic activity could occur following partial hepatectomy in a lobe which had become atrophic as a result of portal diversion. The studies carried out during the present work (Guest et al, 1977) confirm that the two processes of atrophy and of regenerative hyperplasia may occur simultaneously in the same animal: liver mass in the PCT rats appeared to be "hunting" for a new lower level dictated by the available balance of trophic substances, while at the same time the liver remained capable of a hyperplastic response to partial resection which was unimpaired in timing and magnitude compared to normal animals.

Our pair-feeding studies have shown that the greater degree of liver atrophy seen after PCS than after PCT is not simply part

of the generalized loss of body weight in these animals. The difference in relative liver weight between PCS and PCT rats was maintained even when PCT and control animals were "fasted" by the process of pair-feeding to the same body weight as PCS rats. This result further confirms that it is the specific deprivation of portal blood which is responsible for liver atrophy following PCS.

The influence of pair feeding in the brain histology and other studies will be discussed further in Chapters IV and V.

5.3 Structural changes in the liver

The changes seen in PCS animals at 72 hours reflect simply the acute effects of hepatic ischaemia, with a large proportion of animals showing focal hepatic necrosis. Acute damage in PCT and control animals was minimal since hepatic blood flow is interrupted for only a short time. Any "trophic" changes would not be expected to be manifest for some time, although other studies have suggested that these changes are initiated within a few days of shunting (Fisher and Fisher, 1963; Oudea and Bismuth, 1965; Rubin et al, 1965). The most marked change following PCS in our chronic studies was that of fatty infiltration, with healed necrosis evident in only a few animals. These are essentially the same changes as reported by other workers. Others have noted early and specific ultrastructural changes including depletion and disruption of the rough endoplasmic reticulum and deterioration in the appearance of the mitochondria. Our own electron microscopic studies were performed in a very small number of animals, but we also noted

mitochondrial distortion and disruption. The appearance of "light and dark cells" noted here after PCS has also been described by other workers (Rubin et al, 1965). By contrast, the changes on light or electron microscopy in animals with PCT at the same time points were infrequent and of minimal severity.

Nuclear crowding in the PCS animals (a technique used previously by others for the morphological assessment of the shunted liver, Kyu and Cavanagh, 1970) confirmed the subjective suggestion of hepatocyte atrophy, although detailed tracing and weighing procedures were not used (Starzl et al, 1973). PCT animals were normal in this respect. A further interesting observation was that in the PCS animals, with no inflow to the portal venous tracts, the size distinction between hepatocytes of the peripheral and periportal zones of the liver lobules was lost, while this was maintained in PCT animals. Thus it appears that the hepatocytes of the periportal zone continue to enjoy a metabolic "advantage" when the portal tracts are perfused with systemic blood, but that this does not occur when the liver has only its arterial supply. It is impossible to say from the present studies whether this related to the concentration of hormones or nutrients carried in portal venous blood or to differences in blood flow alone. This might be an interesting direction for future work.

5.4 Biochemical liver function tests

Biochemical tests of liver function are notoriously non-specific. The most marked difference amongst the enzymes in these experiments was the gross elevation of AST following PCS,

maximal at 18 hours and thereafter declining towards normal. PCT rats and control animals showed a modest change presumably associated with transient ischaemia during portal occlusion, and reflected also in the small incidence of focal necrosis seen histologically in these groups. In our experience any marked rise in AST following PCT has invariably been related to an operative technical error such as damage to the hepatic artery, and this results in major hepatic necrosis and sometimes in post-operative death. The small number of animals in which this was seen to have occurred were excluded from these studies. The rapid return to near normal levels following the gross AST elevation in PCS animals is a reflection of the marked metabolic reserve of the rat liver. Ozawa and his colleagues (1973) demonstrated that the return of enzymes to normal following portal vein ligation was more rapid in the rat than in the rabbit, and the degree of hepatic reserve so reflected diminished with progression to higher species.

There was a wide range in the magnitude of changes seen in the liver synthesized proteins, albumin and slow $\alpha_1$globulin, but levels following PCS were somewhat lower than those after PCT.

The term "liver function" encompasses a broad spectrum of synthetic, excretory, oxidative and other activities. Although we have demonstrated significant differences between PCS and PCT suggesting that liver function is relatively well maintained following PCT, it would be naive to suppose that this reflects complete maintenance of functional normality in this model. Numerous functions which we have not examined have been shown to

be abnormal following PCS, and since the completion of this work other authors have shown some of these to be affected by PCT also. The effect of PCS of qualitative and quantitative loss of rough endoplasmic reticulum noted above might be expected to be associated with impairment of numerous biosynthetic processes. Pector et al (1980) demonstrated a reduction in cholesterol and lipoprotein synthesis in rats following both PCS and PCT, and prolonged reduction in cholesterol was also seen after PCT in dogs (Castellanos et al, 1981). These effects have been exploited clinically in performing portal diversion for patients with familial hypercholesterolaemias (Starzl, Chase et al, 1973). Hepatic deglycogenation is also seen after both PCS and PCT (Starzl, Marchioro et al, 1965; Starzl, Scanlon et al 1965; Starzl et al, 1969), and this effect has also been utilized in the treatment of glycogen storage disease by portal diversion.

Several enzymes of the hepatic urea (Krebs-Henseleit) cycle have been shown by Reichle et al (1973) to be depressed following PCS in rats. There are probably numerous other synthetic or metabolic processes which are impaired following PCS, and it may be that the common pathway for many of these is loss of activity of the hepatic microsomal mixed-function enzyme system (Rubin et al, 1968). This system is of major clinical importance since it metabolizes a variety of drugs, foreign chemicals and endogenous compounds, whose clearance is therefore impaired in chronic liver failure. Pector and his colleagues (Pector, Ossenberg et al, 1975; Pector, Verbeustel et al, 1975) have in fact demonstrated alterations in hepatic cytochrome P-450 (the terminal oxidase of the mixed function enzyme system) following both PCS and PCT in

the rat. Thus it appears likely that these systems, and many others which use them as a final common pathway, depend upon specific portal substances for their maintenance, and there is accumulating evidence from the studies of Ozawa (1971) and others that insulin may play the key role in this process.

The results of our Rose Bengal studies did not demonstrate any deficit in hepatic excretory function in either PCS or PCT. The dose of Rose Bengal used however was far short of one which would achieve saturation kinetics (Sapirstein and Simpson, 1955), so that subtle differences in excretory function might well be masked in these studies. The relevance of these clearance studies to blood flow is considered below.

Thus although it is clear that there are alterations in liver function following PCT, some of which have not been identified by the present studies, there is a relative maintenance of hepatic mass and function following PCT compared with PCS, and this difference can only be explained by the replacement of portal blood by systemic blood perfusing the portal tracts of the liver. It remains incompletely understood why such differences should arise: the evidence regarding this will be reviewed in Chapter VI. For the present, significant differences have been demonstrated, and it is against the background of these differences that changes demonstrated in central nervous system structure and in other biochemical parameters must be viewed: these changes will be presented in the following two Chapters.

5.5 Gamma globulin changes following PCT

IgG, which is not synthesized by the liver but is filtered by the hepatic reticuloendothelial cells, has been shown to rise to the same extent following both PCS and PCT in comparison with control animals, in which normal ageing changes for this group of rats were demonstrated. This is a different phenomenon from those discussed in the previous section but is of some interest in relation to the aetiology of hypergammaglobulinaemia in patients with chronic liver disease. It would appear from the present studies that the principal factor operative in this model is the shunting of antigen-rich portal venous blood around the liver rather than deterioration in reticuloendothelial cell function. Of course, the true immunological situation in man is more complex than this. These findings have been the subject of two separate publications (Benjamin et al, 1976 and Vaerman et al, 1971), which are included in Appendix C. The mechanisms involved are discussed in more detail in these papers.

5.6 Hepatic blood flow

Hepatic blood flow has been shown to be reduced by some 50% following PCS in the dog, but to be maintained within the normal range following PCT (Heer et al, 1963; Starzl, Lazarus et al, 1962; Baker and Shields, 1970; Kreutzer and Schenk, 1971 and 1972). Studies from our own laboratory measuring hepatic blood flow directly following PCT by means of an isotope clearance technique (Ryan et al, 1978) have demonstrated that total hepatic blood flow remains unchanged up to three weeks after operation. The use of Rose Bengal clearance in the low "tracer"

concentrations described above approximate to the measurement of hepatic blood flow, since even if excretory function is impaired to some extent the levels of Rose Bengal in the plasma do not approach saturation kinetics (see above). This is the basis of the use of dye clearance studies (including Rose Bengal, Indocyanine Green, and colloidal material cleared by the reticuloendothelial cells) for estimation of hepatic blood flow (Benacerraf et al, 1957; Clarkson et al, 1976; Winkler et al, 1965; Areekul et al, 1973).

One group (Chauvaud et al, 1973) have presented results showing no reduction in total estimated hepatic blood flow in the rat 24 hours after PCS, using a continuous BSP infusion technique with hepatic venous catheterization. The same group (Castaing, Beaubernard et al, 1982) found that liver blood flow per gram of liver was the same 14 days after PCS, PCT and PCS with arterialization as in control animals. These findings in PCS rats have not been reported by other groups, and are at variance with the present results and with measured changes in liver blood flow after PCS in other species.

Our Rose Bengal studies underline the importance of careful examination of the kinetics of clearance of such dyes from the circulation. Semilogarithmic replotting of the data from all of these clearance studies would have allowed construction of an acceptably well fitting single regression line through the data points. This approach would have produced a single slope with a k value which is significantly slower for PCS rats than for PCT or control animals, a result which might have suggested impaired hepatic excretion of Rose Bengal. However, using the

biologically and mathematically better fitting model obtained by resolution of the clearance curve into two phases, it became clear that the difference observed between these two groups of animals is entirely in the early (k_1) phase, which is presumed to relate to net hepatocyte uptake of circulating dye, and not in the second (k_2) phase which relates to biliary excretion. Thus the results confirm the reduction in total hepatic perfusion following PCS and its maintenance following PCT, while failing to demonstrate any gross defect in excretory function.

The PCT model in the rat - summary

At this stage we have established parallel models of PCS and PCT in the rat, which differ in the degree of hepatic atrophy and hepatic dysfunction produced, but not in the degree of portal-systemic diversion. While accepting the limitations of this model the distinction between the two preparations is sufficient to allow us to progress to examine other differences and to interpret them in the light of these findings. The next Chapter will examine changes in the central nervous system observed using this model.

CHAPTER IV. HISTOLOGICAL CHANGES IN THE CENTRAL NERVOUS SYSTEM AFTER PCS AND PCT

1. BACKGROUND

The lack of spontaneous coma in rats subjected to end-to-side PCS has been discussed in the previous section. While this clearly casts some doubt on the relevance of rat portal diversion models for the study of PSE, it is clear from behavioural (Campbell et al, 1979; Tricklebank et al, 1978; Beaubernard et al, 1977) and electroencephalographic studies (Herz et al, 1972; Grange et al, 1975) that the central nervous system (CNS) of shunted rats is far from normal. However, the most convincing evidence that specific and reproducible changes are seen in these animals which can be regarded as the analogue of PSE in man and other species lies in histological examination of the CNS. It is relevant firstly to review the nature of the changes observed in man.

1.1 Histological changes in the CNS in man

The first account of the histological changes which we will be examining was given in 1912 by von Hösslin and Alzheimer, who described abnormalities in the nuclei of the astrocytes from a patient with Wilson's disease (at that time known as the Westphal-Strümpell syndrome). Two characteristic changes were noted - gross nuclear enlargement with lobulation and often multiple nucleoli (Type I), and nuclear enlargement with a sharply defined and infolded nuclear rim and a large prominent nucleolus (Type II). These two characteristic changes, which have remained eponymously associated with the name of Alzheimer,

were further defined by Adams and Foley (1949) However, it was more than 50 years after their first description that Victor, Adams and Cole (1965) observed that the changes seen in Wilson's disease are not different from those of PSE, which the authors referred to as "acquired (non-Wilsonian) chronic hepato-cerebral degeneration". Hyperplasia of the astrocytes is not seen in non-specific CNS disorders including anoxia, diabetic acidosis, hypoglycaemia, or drug intoxication. Moreover, their appearance seemed to correlate with the degree of encephalopathy but not with the severity of the hepatic lesion. While other features of CNS damage may be found in chronic liver disease, including degeneration of the nerve cells and myelin sheaths, it is these changes of the astrocytes, and in particular the Alzheimer Type II change, which remain the hallmark of PSE.

In 1966 Kline and his colleagues observed similar changes after experimental PCS in dogs, and at about the same time Doyle (1967) reported these abnormalities in PCS rats. These observations in the PCS rat were soon confirmed and later greatly extended by Cavanagh and his colleagues (Cavanagh and Kyu, 1969). Cavanagh and Kyu (1971) first observed Alzheimer Type I astrocytosis adjacent to cerebral wounds induced in PCS rats, and found that the severity of this process seemed to correlate with the blood ammonia concentration. Zamora, Cavanagh and Kyu (1973) showed by EM studies in PCS rats that the earliest change leading to ammonia-induced Alzheimer Type II astrocytosis is astrocyte swelling, presumed to be a consequence of metabolic hyperactivity. A number of techniques for producing hyperammonaemia have been shown to produce both a clinical

syndrome similar to PSE and typical histology and Alzheimer Type II astrocytosis in rats: Norenberg and his colleagues (1974) used ammonia-liberating cation exchange resins to achieve this.

The astrocyte abnormalities in PSE are not uniformly distributed throughout the CNS. Both in man and in experimental animals the changes are most prominent in areas of deep grey matter including the cerebellar cortex and dentate nucleus, and it is to these areas that most workers have directed their attention. The precise functions of the astrocytes are incompletely understood, but it has become clear that they do not simply form an inert supporting network for the neuronal matrix. Glial cells are highly metabolically active, and are also strategically placed in close juxtaposition to neuronal synapses: it has been shown that in some areas parts of the glial membrane surround the synaptic junction (Peters and Palay, 1965; Henn and Hamberger, 1971). Neuroglial function will be considered further in discussion, but we may note at this point that the astrocytes may take up excess neurotransmitter following synaptic firing, and may also serve a nutritional or supportive role to the neurones, including the regulation of glutamine and glutamate metabolism (Martinez-Hernandez et al, 1977).

There is one particular group of glial cells, the Bergmann cells of the cerebellum, which are regularly affected in both clinical and experimental PSE (Victor et al, 1965; Cavanagh et al, 1972; Diemer, 1976). These cells form a well defined-layer adjacent to the Purkinje cells of the cerebellum. In this situation they have a very high glial cell to neurone ratio (Diemer, 1976), appropriate for supportive cells in this

particularly active neuronal area. These cells regularly show the Alzheimer changes in experimental models, and in carbon tetrachloride treated PCS rats may increase dramatically in number up to a ratio of 25 Bergmann cells to every 2 Purkinje cells (Diemer, 1976, 1978).

1.2 Purpose of CNS studies

The studies described in this Thesis were undertaken to determine whether CNS changes resulting from PCT might differ in character or degree from those expected after PCS. Since in man the development of PSE appears to depend upon impaired hepatocellular function as well as on portal-systemic shunting, it might be expected that better maintenance of hepatic mass and function following PCT should exert a "protective" effect on the CNS despite the presence of shunted portal blood. Accordingly, this Chapter describes the results of histological examination of the CNS, and specifically of astrocyte changes in the dentate nucleus and the Bergmann cell layer, in these two models.

2. EXPERIMENTAL DESIGN FOR CNS HISTOLOGICAL STUDIES

2.1 Experimental groups

(a) Glasgow Study

This was an experiment carried out on animals subjected to PCT, PCS and control operation, sacrificed at 5 weeks. The animals had been fed *ad lib*, housed four to a cage, and weighed and bled at weekly intervals. The results of body and liver weight and biochemical and other changes were described in Chapter III.

(b) Cape Town studies

(i) Acute studies in animals bled at intervals and sacrificed at 72 hours according to the protocol described in Chapter III, Section 3.2.

(ii) A chronic study in animals fed *ad lib*, housed four to a cage and weighed and bled at weekly intervals for 12 weeks according to the previously described protocol.

(iii) A pair-feeding study using the animals also previously described in Chapter III, Section 3.3. Sacrifice in this group took place at 10 weeks, with pair-feeding of controls and PCT animals against PCS animals.

In each of these studies the protocol described for animal handling was exactly as in the biochemical studies in Chapter III, but at the time of sacrifice rapid brain perfusion was undertaken following the laparotomy, as described below.

2.2 Tissue preparation and assessment.

(a) Brain Perfusion

At the time of sacrifice laparotomy was performed under ether anaesthesia, and after inspection of the anastomoses a polyethylene cannula was introduced into the abdominal aorta and loosely tied while blood was withdrawn for biochemical purposes. Following bleeding, and before circulatory arrest occurred, the cannula was advanced up to the arch of the aorta and securely tied in place. The whole liver was then rapidly removed and the blood from the vena cava allowed to flow freely into the abdominal cavity. The aortic cannula was then flushed with saline in order to clear the blood from the tissues of the head, and the saline replaced with an infusion of formal-acetic methanol (40% formalin: glacial acetic acid: absolute methanol, in the ratio 1:1:8). Perfusion with this fluid was continued from a reservoir at physiological pressure for several minutes. During this time the head, neck and upper part of the body rapidly stiffened under the influence of the fixative. Following this procedure the fixed head was severed and placed in a jar of the same fixative until the time of processing.

(b) Preparation of Sections

The brains were carefully removed from the cranial cavity and processed to paraffin in a routine manner. Seven micron sections of cerebrum, brainstem and cerebellum were cut and stained by standard techniques using haematoxylin and eosin, periodic acid-Schiff, and cresyl violet.

(c) Cytological Assessment

All animal heads were numerically coded and were examined 'blindly' by a neuropathologist (in Glasgow Dr. David Doyle, and in Cape Town Dr. Richard Hewlett). Results presented from the Glasgow material are confined to the findings in the dentate nucleus. Using a measuring graticule, high power fields were examined to make a count of at least 400 astrocyte nuclei from each animal. Each nucleus was graded and counted as either "normal" or "abnormal". "Abnormal" cells were defined as those showing one or more of the alterations in nuclear morphology of the Alzheimer Type II. These were:

(i) nuclear enlargement;

(ii) excessive indentation and folding of the nuclear membrane;

(iii) pale staining of the nucleus;

(iv) peripheral dispersion of the stainable DNA.

Results for each animal were expressed as the percentage of astrocyte nuclei thus classified as abnormal.

In the Cape Town studies, quantification of the CNS changes was made by examination of the Bergmann glia. In each animal a number of cerebellar sulci were examined in which the Purkinje cells were seen to have been cut in a uniform plane. All of the Bergmann glia in a high power field were counted and the number demonstrating the characteristic abnormalities of gross nuclear distortion was recorded. A mean of 110 Bergmann cells was examined in each animal (range 71-198).

3. CNS HISTOLOGICAL STUDIES - RESULTS

3.1 Glasgow Series

No abnormalities of the neurones or their processes or of myelination were found in these sections. In addition the proportion of glial nuclei to neurones was assessed in each specimen, and no differences were found amongst the groups. There was no histological evidence of nuclear division in these sections to suggest an increase in the number or local turnover of glial cells as a result of the surgical procedure.

In each area of the brain examined (cerebral cortex, central nuclei, brain stem nuclei and dentate nuclei of the cerebellum), a small number of astrocytic nuclei was found showing some or all of the criteria for abnormality (with the exception of nuclear enlargement, which was not observed in these sections). However, the only area of the brain examined in which there were apparent marked differences amongst the experimental groups was the dentate nucleus of the cerebellum. The percentage of abnormal cells counted in the dentate nucleus in each group is shown in Table 4.1, and an illustrative photomicrograph from a PCS rat can be seen the paper by Doyle et al in Appendix C. The ANOVAR table at the foot of Table 4.1 shows that the percentage of cellular abnormalities was significantly higher in PCT animals than control, but the value for PCS rats was not only higher than control animals, but also significantly higher than that seen after PCT.

TABLE 4.1

Percentage of glial nuclei showing Alzheimer Type II change in the dentate nucleus 5 weeks after operation in Sprague Dawley rats (Glagow Series). At least 400 cells were counted in each animal.

	CONTROL	PCT	PCS
	3.97	12.50	12.75
	3.25	17.40	17.70
	1.98	12.70	23.00
	6.40	16.96	27.00
	3.00	11.97	15.00
	5.60	9.50	
		13.00	
		15.90	
		11.10	
Mean	4.03	13.45	19.09
SE_m	± 0.68	± 0.90	± 2.62

One-way ANOVAR table for above data

Source of variation	Sum of squares	Deg of freedom	Mean square	Variance ratio
Between groups	650.98	2	325.49	26.40 ***
Residual	209.57	17	12.33	
Total	860.55	19		

Detailed comparisons

Control vs PCT	319.07	1	319.07	25.88 ***
Control vs PCS	618.28	1	618.28	50.15 ***
PCT vs PCS	102.33	1	102.33	8.3 *

$p<0.05$ *
$p<0.001$ ***

3.2. Cape Town Series

As noted above, major attention was paid in the Cape Town studies to quantification of abnormalities seen in the Bergmann glia in the cerebellum. However, preliminary examination was also made of the dentate and brain stem nuclei, the basal cell ganglia and the cerebral cortex. No enlargement of the nuclei was found in any of the sections, to suggest inadequate perfusion. No neuronal abnormalities were identified. In counting abnormal Bergmann cells, the only feature accepted as diagnostic (and therefore counted in the statistical evaluation) was the finding of excessive indentation and folding of the nuclear membrane. A number of PCS animals also showed another characteristic of the Alzheimer Type II abnormality, namely peripheral dispersion of the stainable DNA, giving an "open" appearance to the nucleus: while this abnormality was noted, such cells were not counted as abnormal unless they also showed nuclear membrane distortion.

The results for ad lib fed animals in both the acute and chronic studies in Cape Town are shown in Table 4.2. There were no statistically significant differences amongst the groups in the number of abnormal cells by 72 hours after operation. By 10 weeks in this series, there were very numerous abnormalities seen in the PCS rats. The range of abnormal cells in individual PCS rats was 26-95% of Bergmann glia counted, while no PCT animal showed more then 10% of abnormal cells. Analysis of variance shows that this increase following PCS was statistically significant (Table 4.2b). Illustrative photomicrographs from all three groups of chronic rats are shown in Figures 4.1 to 4.3.

Histological material was available for assessment in a small number of animals from the series in which PCT and control animals were pair-fed against PCS rats. In Figure 4.4 the results from these pair-feeding studies are combined with those from the chronic ad lib feeding experiment shown in Table 4.2. It can be seen from Figure 4.4 that the higher percentage of cellular abnormalities in PCS animals persists after pair-feeding of control and PCT rats. Although the numbers in this series are too small for separate analysis, if the results from the ad lib and pair-feeding studies are combined, the differences between the PCS and other groups remains statistically significant.

TABLE 4.2 (a)

Bergmann glial abnormalities 3 days after operation (Cape Town series).
All data are for ad lib fed Long Evans rats.

	C O N T R O L			P C T			P C S		
	Total	Abnormal	%	Total	Abnormal	%	Total	Abnormal	%
Acute	93	3	3.23	88	3	3.41	74	0	0
	78	1	1.28	110	11	10.00	75	4	5.33
	92	2	2.17	88	4	4.55	102	[a]3	2.94
	77	1	1.30	85	6	7.06	78	8	9.20
Mean			2.00			+6.26			4.37
SE_m			0.46			1.46			1.94

[a]Also contained numerous "open" nuclei.

One-way ANOVAR table for above data (acute study)

Source of variation	Sum of squares	Deg of freedom	Mean square	Variance ratio (F)
Between groups	36.10	2	18.05	1.72
Residual	73.51	7	10.50	
Total	109.61	9		

TABLE 4.2 (b)

Bergmann glial abnormalities 10 weeks after operation (Cape Town series).
All data are for ad lib fed Long Evans rats.

	C O N T R O L			P C T			P C S		
	Total	Abnormal	%	Total	Abnormal	%	Total	Abnormal	%
Chronic	86	1	1.16	79	7	8.86	96	44	45.83
	83	1	1.20	71	2	2.82	88	23	26.14
	91	7	7.69	84	3	3.57	82	47	57.32
	87	9	10.34	92	2	9.78	98	79	80.61
							88	84	95.45
							96	68	70.83
Mean			5.10			6.26			62.70
SEm			2.33			1.78			10.18

One-way ANOVAR table for above data

Source of variation	Sum of squares	Deg of freedom	Mean square	Variance ratio
Between groups	11149.62	2	5574.81	15.62 **
Residual	3212.72	9	356.97	
Total	14362.34	11		

Detailed comparisons

Control vs PCT	2.69	1	2.69	0.0007
Control vs PCS	7962.39	1	7962.39	22.31 **
PCT vs PCS	7644.91	1	7644.91	21.42 **

$p<0.01$ **

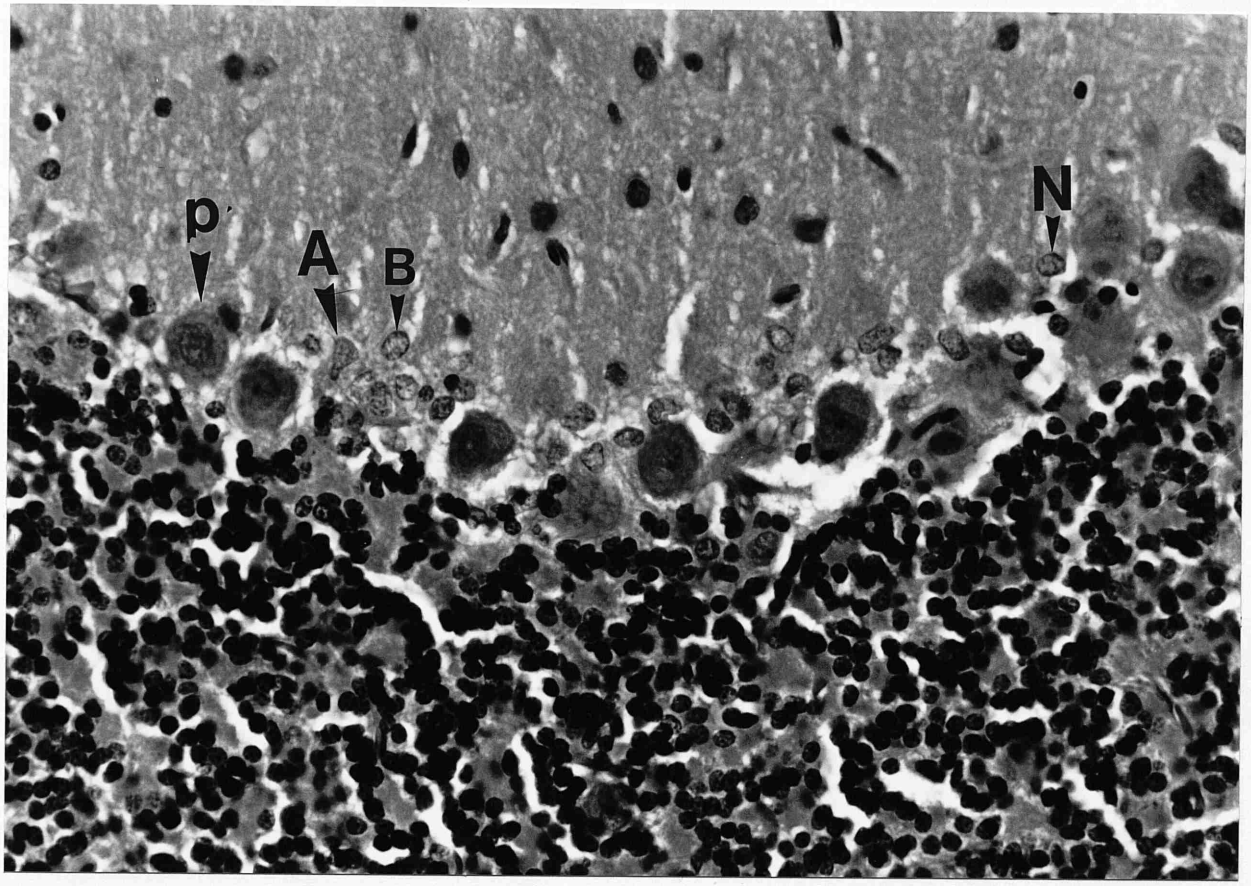


Figure 4.1

Photomicrograph of cerebellum 10 weeks after PCS in an ad lib fed rat.

Normal Purkinje cells can be seen (P), and the layer of Bergmann glia (B) lying between them.

A normal Bergmann cell is marked (N), and is regular and round in shape. Most of the others, however, are abnormal, with polygonal nuclei or irregular protruberances (A).

(Alcian blue stain, magnification X400)

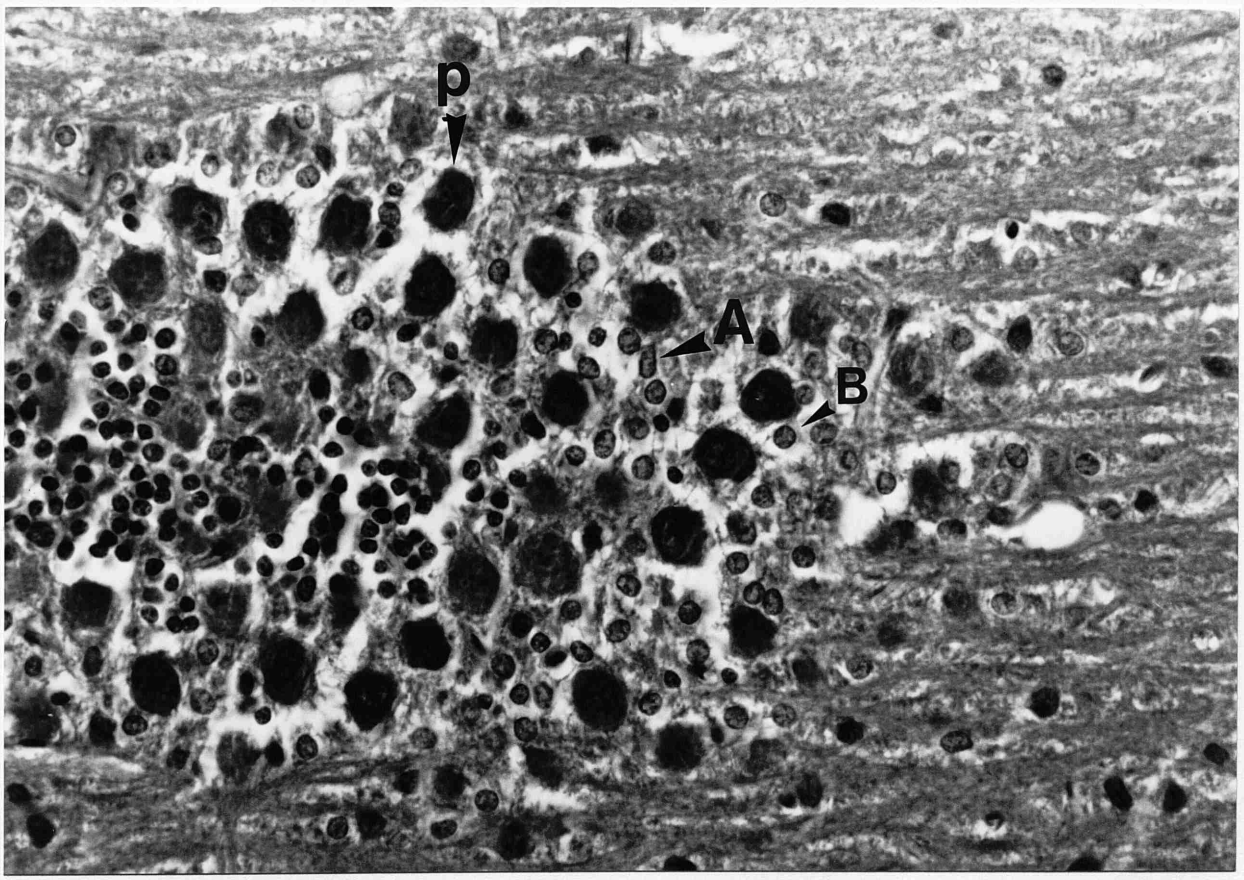
Figure 4.2

Photomicrograph of cerebellum 10 weeks after PCT in an ad lib fed rat.

Compare with Figure 4.1. Again normal Purkinje cells (P) are seen, with their accompanying Bergmann glia (B). In contrast to the appearances after PCS in Figure 4.1, most of the Bergmann nuclei are of normal appearance.

One cell is marked (A) which does show the features of Alzheimer Type II change seen in the majority of Bergmann cells in Figure 4.1.

(Alcian blue stain, magnification X400.)



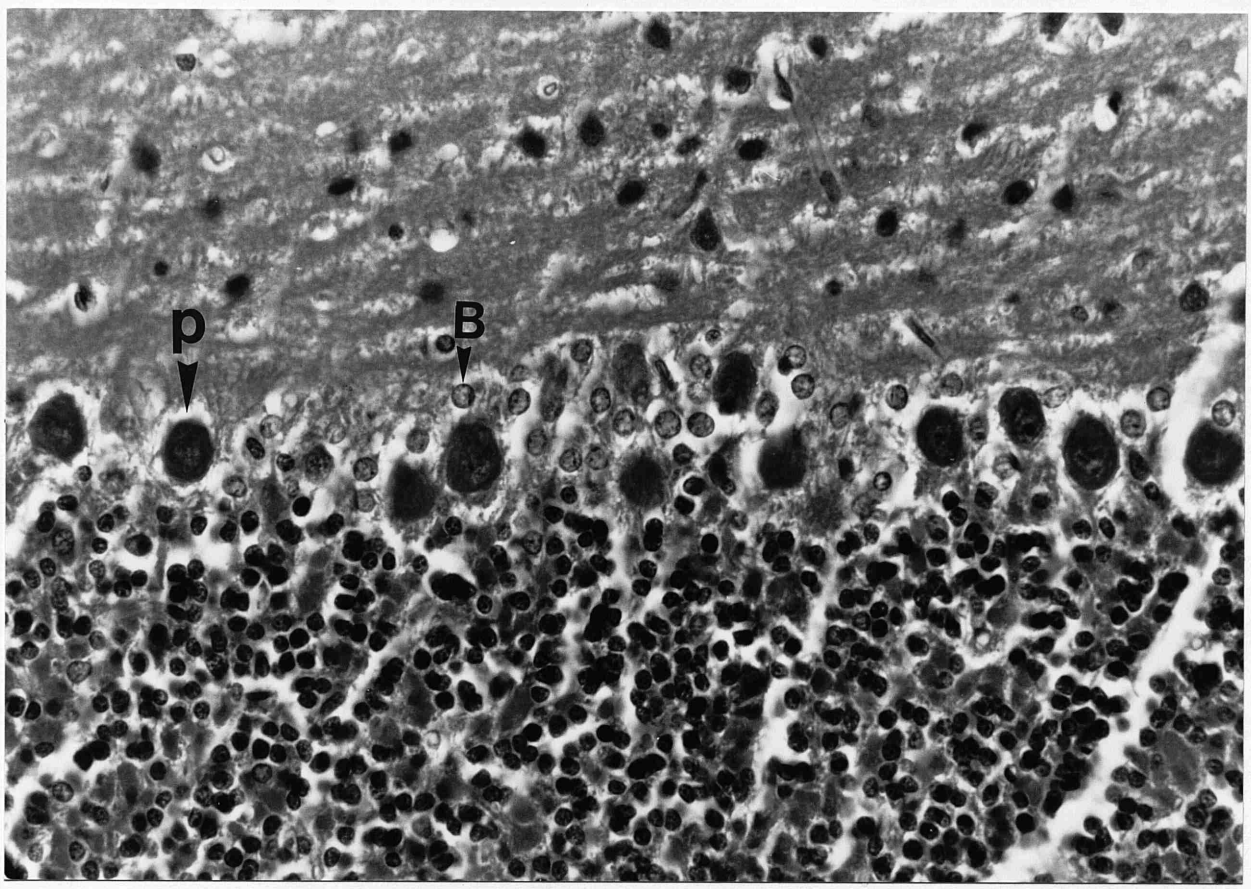


Figure 4.3

Photomicrograph of cerebellum 10 weeks after Control operation in an ad lib fed rat.

Compare with Figures 4.1 and 4.2. Normal Purkinje cells can be seen (P), and the layer of Bergmann glia (B) lying between them. In this section all the Bergmann cells are normal, none of them showing the Alzheimer changes seen in the previous sections.

(Alcian blue stain, magnification X400)

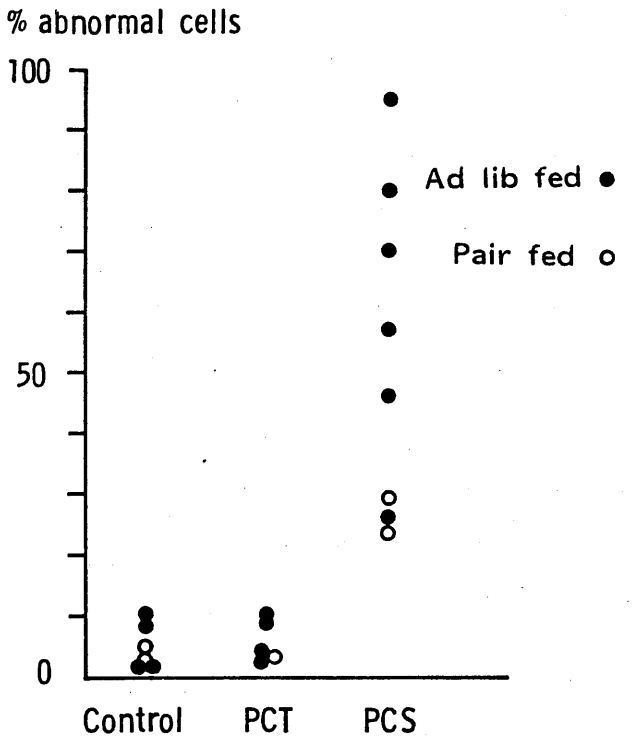


Figure 4.4

The percentage of Bergmann glia showing the Alzheimer Type II change 10 weeks after operation in ad lib fed and in pair-fed animals (ad lib closed circles, pair-fed open circles).

Each point shown is the percentage of cells classified as abnormal in a single rat. The method of counting and the criteria for diagnosing abnormality are described in the text.

4. CENTRAL NERVOUS SYSTEM HISTOLOGICAL STUDIES - DISCUSSION

The findings of the present studies in PCS rats concur with those of numerous other workers. The fact that these classical astrocyte abnormalities are seen to a demonstrably lesser degree following PCT than following PCS is an important observation. If these changes were dependent entirely upon portal-systemic shunting, then they should appear to the same extent following both of these procedures. It is evident that another factor is at work. In the previous Chapter the evidence was reviewed for differences in liver function between the two models. The maintenance of liver blood flow and relative normality of liver mass and function may be the principal factor in reducing the incidence of CNS changes, and if this is so then it is important to examine the reason for this difference more closely.

However, it is also essential to consider other differences between the models which might account for these observations. One possibility is that the nutritional deficit in PCS animals is the cause of the major CNS changes rather than portal-systemic shunting. Our own evidence from the limited pair-feeding experiment would suggest that this is not so. Moreover, changes of the characteristic Alzheimer type have not been described in experimentally induced nutritional deficiency. In rats which have been malnourished from weaning to the age of 42 days, there is a reduction of brain weight which can be reversed on re-feeding (Winick 1974). Histological changes seen during starvation in dogs and in rats have been mostly in the spinal cord or confined to neurones in the central nervous system, with

an increase in oligodendrocytes and a degree of fibrous gliosis, but no changes in the astrocytes. In pigs a protein deficient diet has been shown to result in decreased neuronal and Purkinje cell numbers with swelling of neurones and loss of Nissl granules (Lowrey et al, 1962); astrocyte changes were again not identified.

Chronic hypoglycaemia resulting from liver dysfunction and altered food intake in PCS animals might also be suspected. However, we have observed normal random glucose values with no differences amongst PCS, PCT and control animals in our own laboratory in Glasgow (C.J. Ryan, unpublished observations). Moreover, there was no evidence of hypoglycaemic behaviour or coma in the PCS animals, and hypoglycaemia has also not previously been associated with the specific astrocyte changes described here.

The possibility that some of the changes seen are artefactual must be considered. Many early artefacts in the description of Alzheimer changes resulted from the use of immersion fixation or from a post-mortem delay before fixation. Either of these will result in non-specific cellular swelling including swelling of the astrocytes, and this change probably accounts for a large proportion of the Alzheimer Type I changes described in the literature (Diemer, 1978). It has been suggested, however, on the basis of electron microscopic evidence that even this post-mortem artefact is due to metabolic abnormality of the cells resulting in an abnormal reaction to fixatives and histologic stains (Norenberg and Lapham, 1974). That these changes are not entirely due to increased water

content of the brain has been excluded by EM evidence (Diemer, 1976), although increased cytoplasmic osmolarity may be one factor in astrocyte swelling observed in EM studies (Pilbeam et al, 1983). Increase in astrocyte size is complex and multifactorial, and may in some cases be regarded as a non-specific change. For this reason we have been careful in the present studies not to accept nuclear enlargement or peripheral dispersion of the chromatin alone in classifying the astrocytes as abnormal, but have insisted upon the nuclear folding which may be said to be the one truly characteristic feature of the Alzheimer Type II change (Zamora et al, 1973).

The studies reported here showed no significant incidence of astrocyte abnormalities at 3 days, but well established changes by 5 weeks and 10 weeks. Several workers have studied the evolution of these changes. The classic light microscopic changes following PCS are not normally seen until 4 to 5 weeks after operation (Cavanagh and Kyu, 1971). However, the earliest EM changes are evident at even one week, and consist of swelling of the astrocyte bodies, processes and end-feet. This phase lasts from 1 to 4 weeks, during which time there is an increased infolding of the astrocyte end-feet as they lie against the peri-capillary basement membrane (Zamora et al, 1973). From the fourth to the eighth week these changes of astrocyte swelling are superceded by those of reaction of astrocytic organelles, with changes in the endoplasmic reticulum and proliferation of mitochondria. It is during this phase that the nuclei of the astrocytes become characteristically lobulated and enlarged, with conspicuous enlargement of the nucleolus. Zamora and his

colleagues (1973) have pointed out that the swelling phase of these changes may be quite non-specific, occurring in response to physical brain injury, anoxia, allergic encephalomyelitis, and the effects of drugs such as ouabain and colchicine. However, the reactive changes (as previously noted) are more specific.

The timing of spontaneously occurring changes following PCS has been described above. However, in shunted animals it is possible to induce astrocyte abnormalities at an earlier stage than this by a variety of noxious stimuli. Acute hepatic failure induced by hepatic artery snaring following PCS in dogs led to death in 28 hours, at which time there was an increase in astrocyte numbers and nuclear size (Benson et al, 1970). Shunted rats treated with ammonia exhibit comatose reactions and show histological changes as early as 7 to 12 days (Norenberg et al, 1974; Pilbeam et al, 1983). The severity of the changes may also be affected by dietary manipulation: long-term oral administration of tryptophan in PCS rats led to severe astrocytic changes as well as degeneration of Purkinje cells and reduced neuronal and oligodendrocyte numbers (Bucci et al, 1982). This last study, however, was poorly controlled and may also have suffered from immersion fixation artefacts since some Type I astrocytic changes were observed.

Pilbeam and his colleagues in Australia (1983) reported findings different from those of other workers. While the early astrocyte changes described above were reproduced at 2 weeks after PCS, they declined from 4 weeks onwards, and these workers never found full-blown Alzheimer Type II cells. However, they did observe that plasma ammonia levels, elevated in the early

post-operative period, declined thereafter to levels below those reported by other workers. It is thus likely that these discordant findings are due to hepatopetal collaterals associated with vascular adhesion formation. They were nevertheless able to induce hyperammonaemia and astrocyte changes by feeding of ammoniated cation exchange resins after PCS.

A number of workers have described an increase in astrocyte numbers following PCS. This may be a very early reaction: Gutierrez and Norenberg (1975 and 1977) reported a 30% increase in astrocytes in rat brains after 7 hours of hyperammonaemia induced by methionine sulfoximine administration. Diemer (1976) showed doubling of the astrocyte numbers after 8 weeks of carbon tetrachloride administration in rats. Since there is little evidence of mitosis or amitotic division in the astrocytes, Diemer has suggested that there is a shift of the glial population from oligodendrocytes (which are reduced in number) to astrocytes. He has given careful experimental evidence for this (Diemer, 1978), and has put forward the hypothesis that while the occurrence of Alzheimer Type II cells may correspond to the reversible fluctuating symptoms of PSE, the subsequent loss of oligodendrocytes and later of neurones may be associated with the development of permanent symptoms, and with increased brain sensitivity to ammonia.

PSE and astrocyte function

Despite considerable interest in the glial cells, it remains uncertain whether the astrocyte changes observed represent an attempt by the glia to maintain neuronal homeostasis or a

pathological response to toxins which may contribute to the mechanism of PSE, or perhaps are even a functionally unrelated epiphenomenon. There is no conclusive evidence regarding this, but there is very strong circumstantial evidence arising from the suggested functions of the astrocytes. Astrocytes have been assigned roles in relation to neuronal nutrition, neurotransmitter metabolism, ammonia metabolism, and control of the blood-brain barrier.

The astrocytes have been studied as sources of metabolic and nutritional support for the neurones. An inverse relationship has been observed between enzyme activities in neurones and in adjacent neuroglia. For example, the neurones have a high activity for enzymes of the Krebs cycle (required to liberate the energy needed for synthesis of neurotransmitters and for ionic transport in the nerve cells) and the astrocytes for the oxidative enzymes of the hexose-monophosphate shunt. Thus it has been suggested that the glial cells may present the nerve cells with NADPH, the co-enzyme necessary for the principal reactions of cellular biosynthesis. Thus the symbiotic relationship between neurones and neuroglia originally advanced by Cajal in 1897 appears to be confirmed by modern studies which view the neuroglia as an "auxiliary metabolic unit" for the neurone (Sotelo, 1965). However, this role has been attributed more to the oligodendroglia than to the astrocytes by Friede (1965) since he found that the oligodendroglia had higher activity in relation to oxidative enzyme supply than the astrocytes. Some of this disparity may be due to species differences (Sotelo, 1965).

Quite consistent with the above view of the astrocytes as auxiliary metabolic units is their role in the uptake of excess neurotransmitter. This view has some anatomical support since the glial membrane surrounds the synapse (Peters and Palay, 1965; Henn and Hamberger, 1971). Brain fractionation studies have allowed isolation of fractions rich in either intact neurones or glia for direct biochemical studies on these two cell types. It was shown that the neuronal fraction was more active in protein synthesis, but the glial cells showed a greater ability to accumulate amino acids: the affinity of the glial cell fraction for the CNS transmitter glutamate was 20 times greater than that of the neuronal cells. The glia are also able to inactivate cholinergic compounds due to a high concentration of non-specific cholinesterase, and also have a high specific activity of monoamine oxidase. All of these observations suggest that the glial cell limits extracellular accumulation of transmitters which diffuse out of the synaptic cleft during the transmission of impulses. Since the current view of PSE classifies it broadly as a disorder of neurotransmission, all of this is strong suggestive evidence for a primary rather than a secondary role for the glial abnormalities.

There is evidence that the glia are the only site of ammonia metabolism in the CNS (Williams et al, 1972). It has been noted above that glutamate is an important excitatory neurotransmitter, and that glial cells have a high affinity for this molecule. Glutamate and glutamine are vital components in the control of ammonia metabolism, and glutamine synthetase (which catalyzes the synthesis of glutamine from glutamate and ammonia) has been shown

by immunoperoxidase staining to be localised to the glial cells only (Martinez-Hernandez et al, 1977). Moreover it has been suggested that the inhibitory effects of some biogenic amines in the CNS may be due to the local action of ammonia on glial metabolism (Svaetichin et al, 1965). It is also possible that these same pathways may affect the neuronal environment of K^+ since NH_4^+ ions and K^+ ions traverse CNS membranes in a similar manner (Zamora et al, 1973). Regulation of Na^+ may also be related to these mechanisms (Friede, 1965).

Pilbeam et al (1983b) have studied the fluid and electrolyte content in brain regions 2 weeks after PCS, with and without ammoniated cation exchange resin feeding. They have calculated an increased sodium and chloride space, particularly in the brainstem, suggesting an increased extracellular volume or increased penetration of Na^+ and Cl^- into cells in the brainstem. This finding is consistent with the stimulatory effect of NH_4^+ on membrane-bound Na^+/K^+ ATPase, one of the suggested mechanisms of ammonia toxicity in the CNS (Walker and Schenker, 1970; Zamora et al, 1973). These workers have also suggested that the astrocyte swelling seen in these preparations may be a consequence of cytoplasmic hyperosmolarity due to these ionic redistributions, leading to a "fixation artefact" which in fact has its basis in a real biochemical change.

Finally, the astrocytes <u>may regulate transport</u> across the blood-brain barrier. This supposition is largely based upon anatomical studies demonstrating important surface relationships between the astrocyte end-foot and the pericapillary basement lamina, and on morphological changes seen on electron microscopy

in this region in association with PCS (Zamora et al, 1973). The relationship between blood-brain barrier changes following PCS and the biochemical changes associated with PSE are discussed more fully in the following Chapter.

Knowledge of glial cell function has developed from a state of near-total ignorance before the mid-1960s to be one of the main growth areas of neurobiological research. Of much of the early work, Nicholls (1981) commented that:

> "... the ratio of speculation to sound experimental observation was so high that, apart from continuing contributions from anatomists, to be interested in glia was almost disreputable, somewhat akin to dabbling in parapsychology or memory transfer."

We now have the privilege of "borrowing" from the important advances made by neurobiologists in the last 20 years those known facts about glial function which may be relevant to the pathogenesis of PSE. It is now clear that the astrocytes form a complex cell system in symbiosis with the neurones in the CNS, and may be regarded as an "auxiliary metabolic unit" of wide-ranging functions and of particular importance in neuronal transmission. While it remains speculative to "attempt to relate function to structure and dysfunction to dysstructure" (Conn and Lieberthal, 1979) the proposition that the glial cells play a key role in the mechanism of PSE is a very compelling one.

CONCLUSIONS

We have already noted that the major conceptual difficulty in accepting the PCS rat as a model for PSE is the lack of spontaneous coma. However, the behavioural and electroencephalographic studies referred to previously, along

with the massive evidence that this procedure can produce in the rat morphological changes in the CNS identical to those seen in man suffering from PSE has established the usefulness of the model, and it has gained widespread respectability in some 20 years of experimental practice. We have not yet carried out behavioural studies in PCT animals to demonstrate differences from PCS, but the present histological studies demonstrate a marked reduction in CNS damage following this operation. A single similar study has come to the writer's attention since the completion of the work in this Thesis. Vassannelli et al reported at the European Society for Surgical Research in 1983 the results of an experiment comparing PCS with PCT according to our technique. They commented that "hepatic revascularisation with venous blood .... produced a sharp improvement, as the lesions found were very slight, consisting of a very mild spongiosis involving the cerebellar white matter. Purkinje cells and the granular layer did not present noteworthy lesions. This improvement could not be differentiated quantitatively or qualitatively from that observed with hepatic arterialisation". Although specific comment was not made on the appearance of the astrocytes, which is a surprising omission in this field, their conclusion was similar to that of the present work. In an associated report from the same group (Rigotti et al, 1982) of a study of PCS and liver arterialization "the clinical pattern typically observed in these animals after PCS (bradykinesia, kyphosis, and posterior forced hyperextension), although not specifically evaluated, was shown to be noticeably less relevant after liver arterialisation". These authors also showed similar findings in relation to the relative liver weight after

arterialization to those described in the present work after PCT. Thus in a conceptually similar preparation, these authors have confirmed that CNS damage is less in animals with well maintained hepatic blood flow and mass, in the face of total portal-systemic shunting.

Our studies confirm the suggestion that portal-systemic shunting and diminished liver function are at least co-factors in the aetiology of PSE, a finding which is in keeping with the clinical observation that PSE rarely occurs in the setting of normal liver function. Thus even in the presence of a total portal-systemic shunt, major liver dysfunction seems to be required for CNS derangement to occur. Conversely we may suggest that maintenance of liver function protects the CNS against the effects of portal-systemic shunting. The next step, which is described in the following Chapter, was to examine some of the biochemical changes in the plasma and the brain in these same models, in order to determine which of these might show a similar relationship, and possibly to cast further light on the pathogenesis of PSE.

CHAPTER V. BIOCHEMICAL CHANGES ASSOCIATED WITH PORTAL DIVERSION

1. BACKGROUND

It is clear that in the broadest terms we must seek a chemical mechanism for the pathogenesis of PSE. A wide variety of biochemical abnormalities have been proposed as causative or contributory to the production of the syndrome, and the principal of these are enumerated in Table 5.1. In introducing the biochemical studies which we have performed in PCS and PCT rats, these agents will be briefly discussed in order to lend perspective to the direction pursued in the present studies. However, detailed discussion of the biochemical mechanisms will be reserved until Section 4. This is partly to allow discussion of our own results in this context, and partly because a number of important advances have been made since the completion of this work, and these must be carefully reviewed.

1.1 Loss of a "hepatic factor"

It is not surprising in view of the major synthetic functions of the liver that it was early proposed that deficiency of a substance synthesized by the liver was primarily responsible for the CNS disturbances of liver disease. In the 1950s it was suggested that lack of cytidine and uridine might be responsible for PSE (Geiger et al, 1954; Geiger, 1958). This hypothesis was based on improved survival of an isolated perfused cat brain following the addition of cytidine or uridine to the perfusion fluid. While Geiger's prepartion has not been used in similar studies since that time, cross-circulation experiments in rats

TABLE 5.1

Biochemical agents implicated in the pathogenesis of PSE

Loss of liver factor(s), e.g. cytidine, uridine
Ammonia
Short chain fatty acids
Methionine, mercaptans
Tryptophan
Aromatic amino acids
False neurotransmitters
GABA
Acetylcholine
Phenols
"Middle molecular weight compounds"
VIP

did not confirm this hypothesis (Roche-Sicot et al, 1974). These latter studies showed that systemic to portal cross-circulation of hepatectomized rats with normal donors was more effective than systemic to systemic circulation, which would not be the case if the primary mechanism were absence of a substance synthesized by the liver. These cross-circulation studies are part of a large body of evidence suggesting that it is the detoxifying or clearing aspect of liver function which is primarily deficient in PSE. Nonetheless, the isolated brain/liver perfusion preparation should be a valuable one in this field, and it is the writer's personal view that it remains of considerable importance to confirm and extend Geiger's observations.

1.2. Ammonia

More than two decades ago Walshe stated "if the role of ammonia in the genesis of hepatic coma is not yet satisfactorily defined, there is no other theory that comes so near to conforming to the observed facts" (Walshe, 1953). Despite the enormous amount of work during the last 20 years this statement remains broadly true. The evidence for any putative agent in PSE rests in its fulfilment of the several essential criteria:

(a) it is produced by degradation of nitrogenous material in the gastrointestinal tract;

(b) it is extracted and metabolized by the liver;

(c) elevated plasma levels are found in chronic liver disease and in particular in the presence of PSE;

(d) it has access to the brain;

(e) it may exert toxic effects in the nervous system.

In relation to ammonia we will examine each of these in turn.

(a) The major source of production of ammonia is the gastrointestinal tract, mostly from bacterial action on nitrogenous material in the colon, though in chronic liver disease there may also be significant production from the small intestine due to colonization with faecal flora (Martini et al, 1957). Ammonia levels rise promptly in man Following ingestion of dietary protein or urea (McDermott et al, 1954). It may be that meat protein is more potent than vegetable protein in this respect (Greenberger et al, 1977), but blood appears to be gram for gram the most potent substrate (Bessman and Mirick, 1958). It is clear that there are sources of ammonia absorption from the gastrointestinal tract independent of bacterial degradation, since elevated ammonia levels were found equally following PCS in germ-free rats (Jeppsson et al, 1979) and dogs (Nance and Kline, 1971). (The kidneys are also a source of ammonia production, as a by-product of the excretion of hydrogen ion: this source is probably quantitatively unimportant in the present context.) An intestinal source for any potential neurotoxin is important since many of the empirical measures in the management of chronic PSE involve purgation of the large bowel, alteration of its bacterial spectrum by oral antibiotics, or in the most extreme cases surgical colonic exclusion. All of these, and in addition the use of lactulose, the functions of which are multiple and incompletely understood, are uniformly effective in reducing absorption of ammonia and blood ammonia levels, and in favourable circumstances may ameliorate PSE (Conn and Lieberthal, 1979).

(b) The liver is a major site of detoxification of ammonia, and as much as 80% of portal venous ammonia is removed by the liver, so that the highest and lowest ammonia concentrations in normal man are found in the portal vein and the hepatic veins respectively (Duda and Handler, 1958). There is also a quantitatively small uptake of ammonia by the brain, an important phenomenon which will be discussed more fully later (Bessman and Bessman, 1955). Skeletal muscle also consumes ammonia at rest, but produces large amounts during vigorous exercise (Conn and Lieberthal, 1979). Muscle wasting in cirrhosis may deplete a significant site of ammonia utilization.

Hepatic ammonia degradation is achieved mainly by incorporation into carbamyl phosphate which then enters the urea cycle by combining with ornithine to form citrulline, and thereafter urea (see Figure 5.1). The liver appears to be the only organ in which carbamyl phosphate and urea can be synthesized. Measurement of the maximal rate of urea synthesis has been used as an index of hepatic function.

Amination of α-ketoglutarate to glutamate and thence to glutamine (Figure 5.2) is the primary means of detoxifying ammonia in the brain and kidney, but appears to play a minor role in the liver.

(c) We have already noted in the historical review (Chapter II) that elevation of plasma ammonia in chronic liver disease has been observed for very many years, first in animals and then in man. Moreover, it has been widely demonstrated that administration of ammonium salts, or other substances which

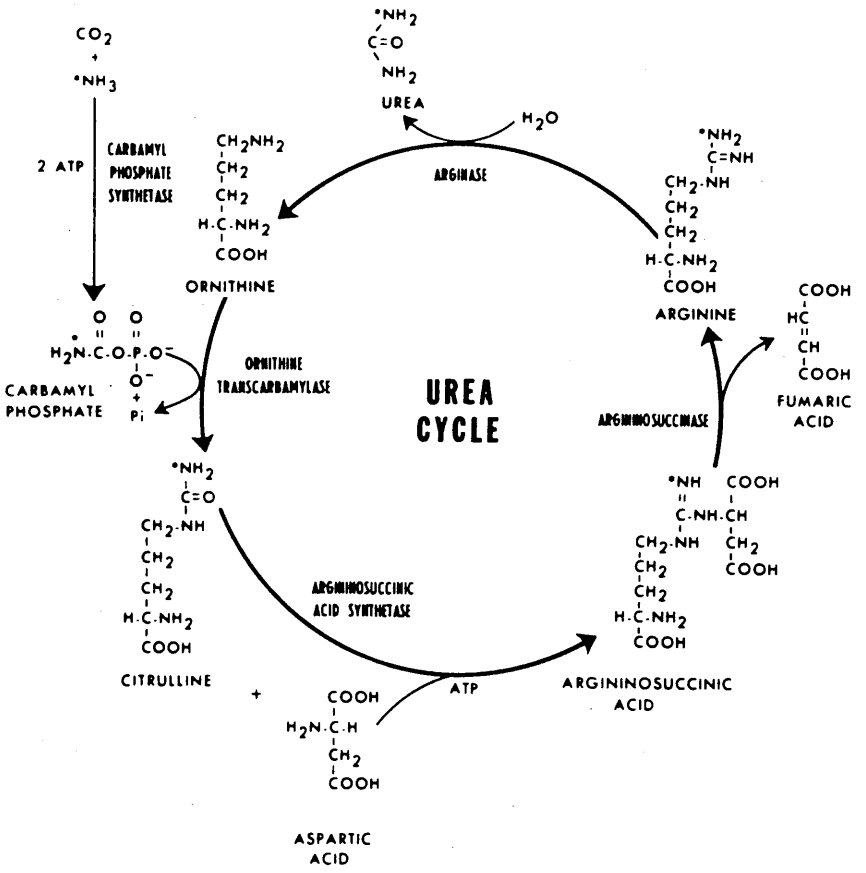


Figure 5.1

The urea (Krebs–Henseleit) cycle.

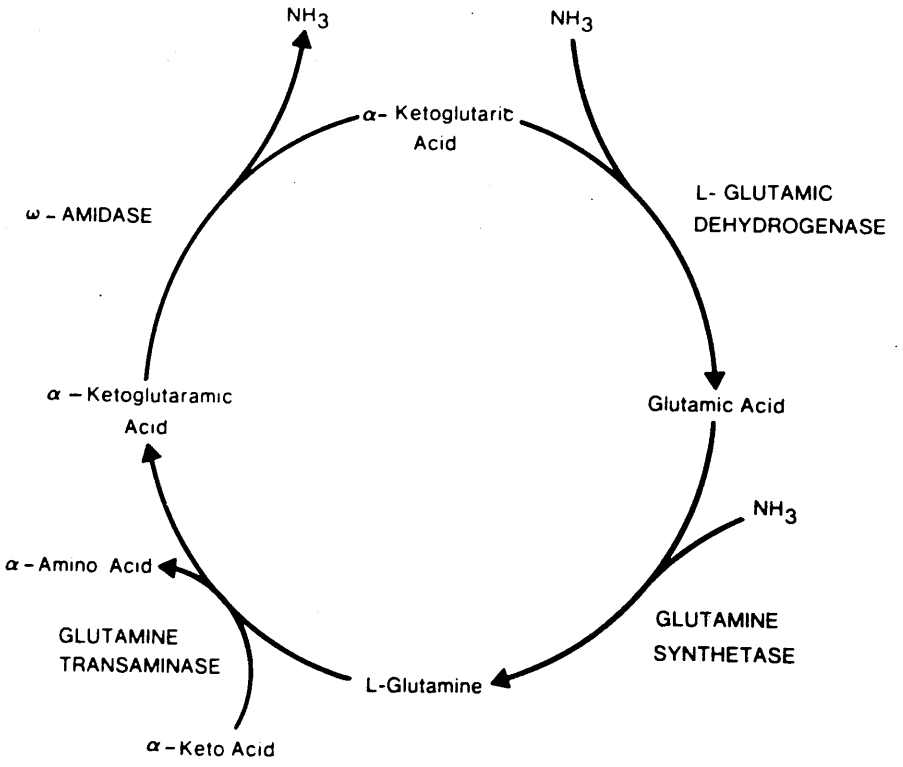


Figure 5.2

The metabolism of ammonia, glutamate and glutamine in the brain. Ammonia is incorporated into α-ketoglutarate and glutamate to form glutamine. Glutamine is transaminated to α-ketoglutaramate, which is deaminated to complete the cycle.

liberate ammonia, to patients with liver disease or with portacaval anastomoses results in production or aggravation of symptoms of PSE (White et al, 1955; McDermott and Adams, 1954; Summerskill, 1955; and many others). However, while many authors have reported a correlation between blood ammonia levels and the degree of PSE (White et al, 1955; McDermott et al, 1954; Sherlock et al, 1954) others have reported a rather poor and inconstant correlation between these two (Zeegen et al, 1970; Stahl, 1963). Blood ammonia has been reported normal in severe encephalopathy (Phillips et al, 1952), and even in Grade IV coma (Knell et al, 1974). Arterial estimations are more specific than venous, but may still be normal in some 10% of patients in hepatic coma (Summerskill and Wolfe, 1957; Phear et al, 1955). CSF ammonia is more often elevated than blood ammonia. However, despite its inconstant relation to the syndrome, estimation of plasma ammonia remains a widely performed investigation in patients with suspected hepatic encephalopathy. The problems of this inconstant relationship between ammonia and PSE may be due to a number of causes. Firstly, not all PSE is induced by excessive nitrogenous loads, and one might not expect changes in blood ammonia when PSE is precipitated or aggravated by hypoglycaemia, acid-base or electrolyte imbalance, or excessive use of sedatives. Secondly venous ammonia levels, as opposed to arterial, are highly susceptible to fluctuations related to muscular exercise (Conn and Lieberthal, 1979). Furthermore, the temporal relationship between ammonia levels and estimation of the level of PSE and the subjective nature of the assessment of the latter may lead to further disparity. However, it must still be accepted that the relationship between plasma ammonia levels

and PSE over a very considerable period of investigation has remained inconclusive.

(d) Ammonia traverses most tissue interfaces readily, and easily enters the CNS. Transport across cell membranes depends upon non-ionized ammonia in solution, which constitutes 1-3% of the total at pH 7.4. At the pH of intracellular fluid (< 7.0) a much smaller fraction exists as ammonia, lowering the partial pressure and encouraging diffusion from extracellular into intracellular fluid. Anything therefore which increases the pH gradient between the extracellular and intracellular compartments will favour the passage of ammonia into the cells: this would explain for example the increased toxicity of ammonia in metabolic or respiratory alkalosis.

(e) At levels normally measured in hepatic coma ammonia does not appear to be directly toxic to the CNS (Schenker et al, 1967; Cole et al, 1972). It thus appears likely that ammonia toxicity is related to metabolic events consequent upon its detoxification in the CNS, and not to its cerebral concentration per se. Ammonia may deplete the energy stores of the CNS by diverting Krebs cycle intermediates such as α-ketoglutarate into synthesis of glutamate and glutamine (Bessman and Bessman, 1955). Counter to this suggestion is the observation that in man blood and CSF levels of α-ketoglutarate are usually increased rather than decreased (Summerskill et al, 1957). Moreover, α-ketoglutarate and high-energy phosphate levels in the CNS were not reduced in animals with hepatic coma (Hindfeld and Siesjo, 1971; Biebuyck et al, 1975; Hindfeld, Plum and Duffy, 1977). It has also been proposed that ammonia leads to the accumulation of

γ-aminobutyric acid (GABA) (Goetchus and Webster, 1965). Once again, however, Biebuyck and his colleagues (1975) found normal levels of GABA in PCS rats following hepatic artery ligation. The importance of GABA in PSE is further considered below. A third possible toxic effect is the transamination of glutamine in the presence of excess ammonia to α-ketoglutaramate, a false neurotransmitter. This will also be considered below.

The possibility that ammonia inhibits acetylcholine synthesis is rendered unlikely by the finding of normal acetylcholine levels in the cortex and brainstem in ammonia intoxicated animals (Walker et al, 1971).

Finally, in relation to the evidence for ammonia as the primary agent in PSE it should be noted that several constant metabolic features of hepatic coma have not been reproduced by ammonia intoxication. Serotonin is greatly elevated in the brains of rats in hepatic coma as well as in the CSF of patients with Grade IV encephalopathy (Fischer and Baldessarini, 1975; Knell et al, 1974). However, ammonia given to experimental animals in concentration greater than that seen in man failed to change cerebral serotonin (Walker et al, 1971).

Further clinical evidence against a prime role for ammonia is seen in children with inborn defects of the urea cycle enzymes resulting in marked hyperammonaemia, in whom the clinical picture (a mildly encephalopathic, hyperkinetic preconvulsive state followed by seizures and post-ictal coma) differs from that of chronic PSE (Conn and Lieberthal, 1977; Flannery et al, 1982). Moreover, visual evoked potentials elicited in laboratory animals

with hyperammonaemia differ from those seen in experimental hepatic encephalopathy (Pappas et al, 1982).

It remains clear that while the role of ammonia as a sole causative agent has lost its appeal over 30 years of investigation, it is still probable that it occupies a central role in some of the metabolic derangements of PSE. Its pungent odour may continue to permeate this area of research for some time to come.

1.3 Fatty acids

Short chain fatty acids are known to be increased in the blood in chronic liver disease. In liver disease without coma values of about twice normal have been observed, while in hepatic coma they may rise to five times normal (Zieve, 1966). However, these observations were not accompanied by a good correlation between the depth of coma and the plasma level of fatty acids. Moreover, fatty acids of this chain length have been administered to patients with cirrhosis without any undue cerebral effects (Morgan et al, 1974).

However, Zieve and his associates have suggested that the effects of fatty acids are only produced in synergism with ammonia (Zieve et al, 1974). These workers observed that large doses of ammonium salts supplemented with short chain fatty acids would render normal animals comatose. Fatty acids in this setting appear to cause disproportionately large increments in blood ammonia, possibly by inhibition of urea synthesis and glutamate dehydrogenase activity. Moreover, short chain fatty acids displace tryptophan from its binding site to albumin

(Curzon et al, 1973), and increased entry of tryptophan to the CNS may be important in PSE (see below). Certainly short chain fatty acids are able to reproduce experimentally many of the features of PSE, although the levels of ammonia observed in these experimental models are many times higher than those seen clinically (Fischer and Baldessarini, 1975). Short chain fatty acids also fail to satisfy some of the "postulates" put forward above: in particular it is not easy to relate them to dietary protein or to other stimuli known to provoke or exacerbate PSE. There remains little evidence to support their role in the syndrome.

1.4 Methionine and mercaptans

Methionine has long been known to have the effect of inducing PSE in cirrhotic patients, and these effects are out of proportion to the increase in ammonia level resulting from the slow liberation of ammonia from methionine (Webster and Gabuzda, 1957). Methionine is metabolized to methanethiol (methyl mercaptan), probably in the gastrointestinal tract, and the mercaptans formed by this process are further metabolized in the liver. Foetor hepaticus, the characteristic breath odour of patients in liver failure, has been shown to be due to mercaptans (methanethiol and ethanethiol) and dimethyl sulphide (Chen et al, 1970). While a causal relationship is difficult to demonstrate, Zieve and his colleagues (1974) have in fact shown that each of these three sulphur compounds is individually cerebrotoxic in experimental animals, and furthermore that they may potentiate other suspected comagenic agents, and in particular ammonium chloride and short chain fatty acids. Methanethiol is 20 times

as toxic as ethanethiol, and 60 times as toxic as dimethyl sulphide. These workers suggested that ammonia may inhibit the detoxification of methanethiol to dimethyl sulphide, and this synergistic toxicity between ammonia and methionine has been confirmed in dogs with portacaval shunts (Merino et al, 1975). It is interesting that the studies of Greenberger and his colleagues (1977) showed that cirrhotic patients maintained on a vegetable diet suffered less encephalopathy than those on an animal protein diet, and that the methionine content of the former was one third to one half of the latter. There may of course be other reasons for this difference.

Recent studies from Al Mardini and his colleagues in Newcastle (1984) showed much higher levels of blood methanethiol in encephalopathic patients than in control subjects or subjects with liver disease who were not encephalopathic. Ethanethiol and dimethyl sulphide concentrations were similar in the three groups. However, blood levels of methanethiol were not clearly related to coma grade and a considerable range of responses was seen in rats injected with intraperitoneal methanethiol. Moreover, the concentration of methanethiol associated with coma in animals was at least 10-fold greater than in patients with hepatic encephalopathy, and also brain concentrations were similar in comatose rats and those which remained awake. Moreover, recent work using visual evoked potentials in rabbits showed fundamental differences between changes produced by the synergistic effects of ammonia, mercaptans and fatty acids and those seen in PSE (Jones, 1983).

1.5 Tryptophan

Numerous workers have demonstrated a relationship between tryptophan administration, elevated plasma tryptophan levels, and PSE or fulminant hepatic failure (Knell et al, 1974; Ono et al, 1978). Knell also found increased concentrations of 5-hydroxy indoleacetic acid in the spinal fluid of patients with PSE or fulminant hepatic failure. Feeding of tryptophan in a dose of 200 mg/kg/day had no effect upon normal rats, but induced weight loss, toxic hepatitis and behavioural changes in PCS rats (Bucci and Chiavarelli, 1979). The authors same group (Bucci et al, 1982) also demonstrated astrocyte changes in the dentate nucleus along with degeneration of Purkinje cells and decreased oligodendrocyte and neuronal numbers. Close examination of this work, however, suggests that some of these changes were artefactual (or of the Alzheimer Type I variety) due to immersion fixation.

Tryptophan is the only amino acid which is bound to albumin in the blood, and only the free fraction of tryptophan is available for exchange across cell membranes, or for entry into the CNS (Korpi and Oja, 1978). However, the entry of tryptophan into the brain is not a simple process, and the elevation of brain tryptophan in liver disease is not fully explained by the increase in plasma free tryptophan (Bloxam and Curzon, 1978): although both brain and plasma free tryptophan are elevated from one day after PCS in the rat, the brain to plasma ratio remains disproportionately elevated. Tryptophan and the other large neutral amino acids compete for entry across the blood-brain barrier by way of common carrier-mediated mechanisms (Fernstrom

and Wurtman, 1972; Orlowsky et al, 1974), and brain tryptophan may increase as a result of a fall in plasma branched-chain amino acid levels. The implications of this competitive process are crucial, and will be discussed in more detail below.

Given the increase in brain tryptophan seen in liver disease or after PCS in experimental animals, how may this exert toxic or comagenic effects? Tryptophan is converted in the brain to 5-hydroxy tryptophan and 5-hydroxy tryptamine (serotonin, see Figure 5.3). Thus increased tryptophan levels may stimulate serotonin synthesis, and indeed increased serotonin levels are found in the brain following PCS (Baldessarini and Fischer, 1973). Increased 5-HIAA levels suggest that synthesis and turnover of serotonin may be increased to an even greater extent than serotonin concentration (Knell et al, 1974). Serotonin, an inhibitory neurotransmitter, may replace endogenous neurotransmitter and induce abnormal nervous activity. This has also led to the use of L-DOPA as a therapeutic measure in hepatic coma (Colon and Sandberg, 1973). The concept is that L-DOPA readily traverses the blood-brain barrier and is converted to dopamine, which as a more effective neurotransmitter displaces the high levels of serotonin, and thus improves neurological function. Broader aspects of the false neurotransmitter hypothesis will be discussed below.

1.6 Amino acids

Although abnormalities of plasma amino acids (particularly tyrosine) have been recognized for many years, the first systematic report of amino acid abnormalities in PSE was that of

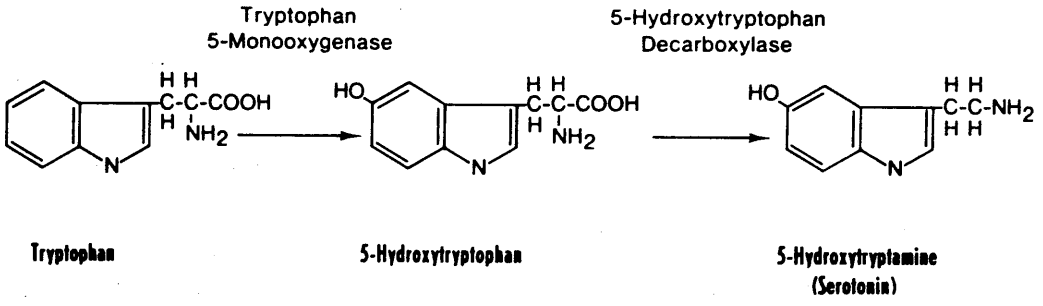


Figure 5.3

The metabolism of tryptophan in the brain.

Walshe in 1953. He established a pattern in acute liver injury which with a few variations has been confirmed by all subsequent workers, namely elevation of phenylalanine (PHE), tryptophan (TRY), tyrosine (TYR), methionine (MET), aspartate (ASP), proline (PRO) and cystine (CYS). Valine (VAL), leucine (LEU) and isoleucine (ILEU) were present in normal concentrations. The principal amino acids of interest in discussion of PSE are the large neutral amino acids. These are shown in Figure 5.4 with their chemical structures and the abbreviations to be used in the text.

The recent resurgence of interest in amino acids in PSE was stimulated by the work of Fischer, first at the Massachusetts General Hospital and subsequently in Cincinnati, Ohio. These workers demonstrated that patients with PSE had levels of MET, PHE and TYR two to three times normal, with similar elevations of ASP and GLU. They also demonstrated that VAL, LEU and ILEU were decreased to about half normal (Rosen et al, 1977). Important studies of dogs with PCS and experimental PSE have shown a similar pattern of amino acids in the plasma (Smith et al, 1978), which may be summarized as an elevation of the aromatic amino acids (AAA) and a fall in the branched chain amino acids (BCAAs). In addition the AAAs are found to be increased in CSF, while CSF BCAAs remained normal.

It is important to note in passing the difference between the amino acid pattern seen in chronic liver disease and PSE and that in fulminant hepatic failure. The largest series of the latter type of patients was recorded in Kings College Hospital, where Record and his colleagues (1976) found that all the amino

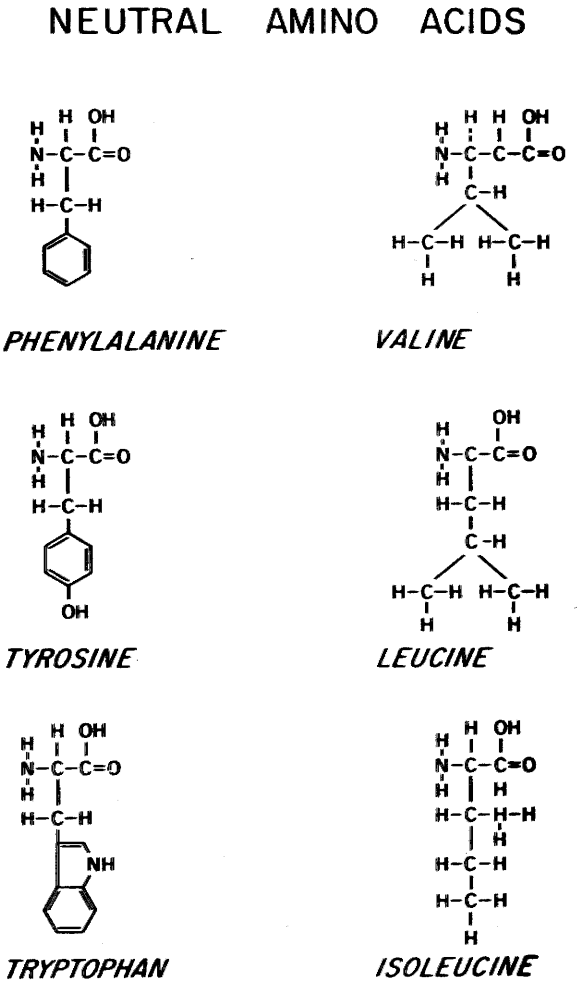


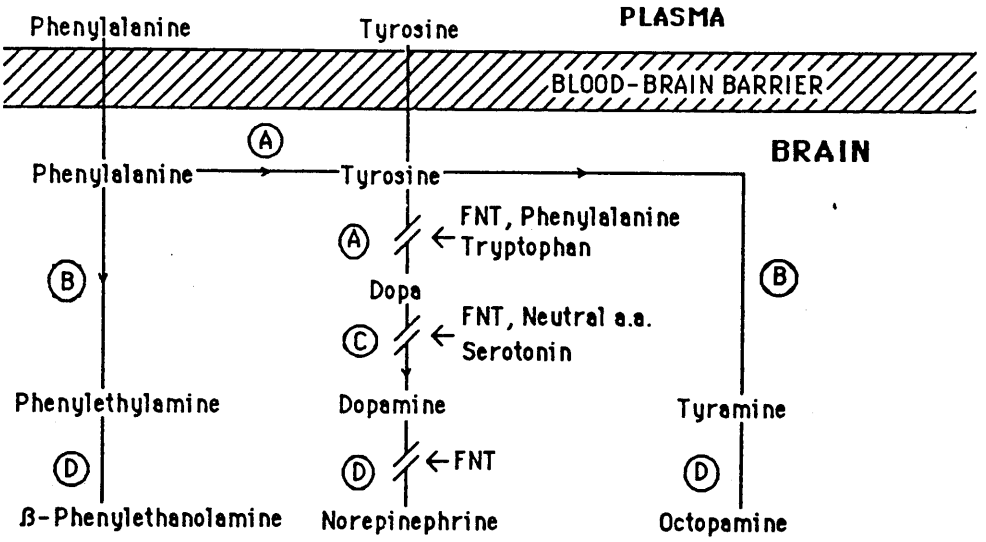
Figure 5.4

The large neutral amino acids of relevance to these studies.

acids except the BCAAs were elevated, with maximal increments in the concentrations of MET, PHE and TYR (MET being 30 times normal). This generalized increase is possibly related to non-specific release of amino acids during necrosis of hepatocytes, and this supposition is supported by a close linear relationship between elevation of AST and that of plasma TYR in patients in fulminant hepatic failure (Rosen et al, 1977).

The pattern of amino acid abnormalities described is seen in chronic liver disease with or without PSE. Conn and his colleagues have suggested that portal-systemic shunting, whether due to the liver disease or surgically produced, is almost a prerequisite for these changes in their fully developed form (Conn and Lieberthal, 1979 p.101-102). Fischer and his colleagues found recognizeable quantitative differences between patients with and without PSE. They examined the molar ratio of BCAAs to AAAs ((VAL + LUE + ILE)/(PHE + TYR)) the value of which is normally 3.0-3.5 (Fischer et al, 1974). In PSE they found that this ratio fell to well below 1.0, and concluded that this might be critical in the induction of PSE and that there may be therapeutic value in attempts to restore the ratio to normal. Moreover, when this group examined the molar ratio in fulminant hepatic failure they found that it was even lower, and also resistant to "normalization" by administration of balanced amino acid solutions, in contrast to patients with chronic PSE (Fischer et al, 1976).

Elevation of the AAAs may be readily explained on the basis of their normal route of metabolism (Figure 5.5). PHE is hydroxylated to TYR which is then transaminated, and the enzymes



A = tyrosine hydroxylase; B = aromatic amino acid decarboxylase; C = dopa decarboxylase; D = dopamine ß-oxidase; FNT = ß-phenylethanolamine.

Figure 5.5

The metabolism of the aromatic amino acids phenylalanine and tyrosine in the central nervous system.

involved in both of these reactions are almost exclusively hepatic in location. The fall in BCAAs is less clearly explained. BCAAs are metabolized in peripheral tissues, including muscle and adipose tissue, where they may be used as an energy source under the metabolic control of insulin. That the BCAAs are reduced secondary to the hyperinsulinism present in patients with liver disease, or in patients or experimental animals following PCS, is an important hypothesis (Munro et al, 1975, Sherwin et al, 1978). Hyperglucagonaemia is also seen in liver disease and PCS (Sherwin et al, 1978). While it is probable that these alterations in insulin and glucagon metabolism may be of profound importance in the determination of amino acid balance and in the pathogenesis of PSE, it remains uncertain whether the main determinant of hyperinsulinaemia and hyperglucagonaemia is shunting of blood around the liver or hepatocellular dysfunction. This key question will be discussed later.

It has already been noted that the large neutral amino acids share a common mode of entry into the CNS, and that there may be competitive relationships amongst their rates of transfer (Fernstrom and Wurtman, 1972; Orlowsky et al, 1974). Thus the "amino acid-neurotransmitter hypothesis" claims that the disturbed amino acid pattern found in PSE is responsible for changes in concentration of neurotransmitter precursors which lead to an altered physiological state of the CNS.

1.7 False neurotransmitters

Numerous neurotransmitter abnormalities have been reported

in patients and in animals with PSE. Serotonin cahnges have already been referred to (Fernstrom and Wurtman, 1972), and increased TRP transport noted (Curzon et al, 1973). Acute hepatic coma produced by PCS and hepatic artery ligation resulted not only in decreased levels of norepinephrine (a putative adrenergic neurotransmitter), but also in markedly increased levels of octopamine, a substance with weak neurotransmitter function (Dodsworth et al, 1974; Fischer and Baldessarini, 1971). In addition β-phenylethanolamine, a β-hydoxylated false neurotransmitter, is found in high concentration (Fischer and Baldessarini, 1971; Fischer and James, 1972; Aguirre et al, 1974). Other important observations made by these workers were that a good correlation existed between urinary excretion and blood levels of octopamine in patients with PSE. Elevated levels of octopamine occurred in the brain before any rise of serum levels, suggesting that the accumulation of these substances is occurring as a result of brain metabolism. The way in which these β-hydroxylated false transmitters are formed as an end-stage of PHE and TYR metabolism in the CNS is shown in Figure 5.6. It can be seen that these changes could be driven by excessive transport of AAAs into the CNS as a result of an imbalance between the AAAs and BCAAs in the plasma. Fischer's group have demonstrated that although under normal circumstances TYR will proceed to catecholamine production, excessive levels of brain TYR result in the formation of octopamine, and that there is a strong correlation between these two substances in PCS rats (James, Hodgman, Funovics and Fischer 1976). Increased PHE levels, as well as predisposing to the formation of β-phenylethanolamine, may also compete with TYR for the enzyme

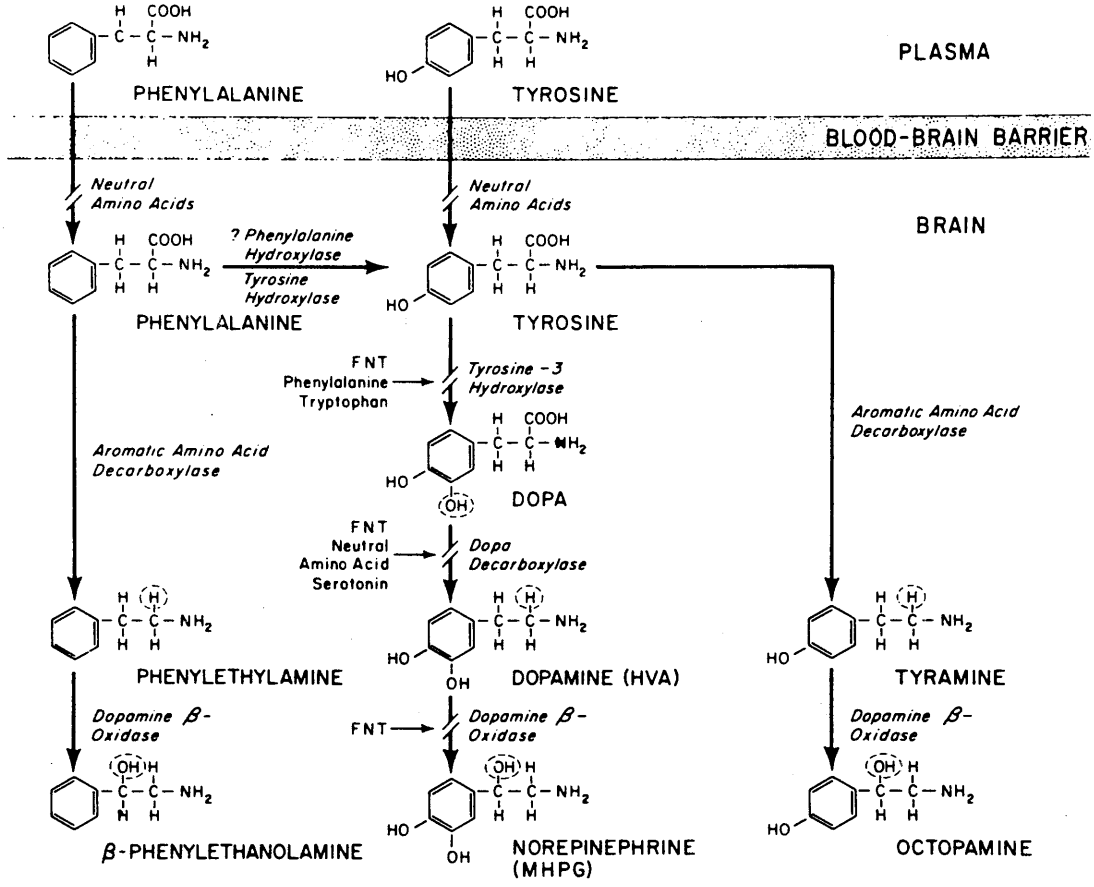


Figure 5.6

The metabolic origin of false neurotransmitters in the central nervous system, as in Figure 5.5, showing the structures of the molecules.

tyrosine hydroxylase, since TYR and PHE have similar affinities for this enzyme. In addition TRP, as well as increasing brain serotonin, further reduces catecholamine synthesis by inhibiting the hydroxylation of TYR to DOPA.

The relative importance of the ratio of neutral amino acids and of the ratio of free to bound TRP in the plasma in explaining increased brain TRP and contributing to the effects described above is uncertain. Fischer's group have emphasized the importance of the amino acid ratio, largely on the evidence that administration of BCAAs alone have produced a decrease in brain TRP (Cummings et al, 1976).

Other groups have also contributed to the false neurotransmitter debate. Sherlock and her colleagues (Manghani et al, 1975) showed that increased blood levels of dopamine in PSE correlated approximately with the degree of coma. Lam et al (1973) also demonstrated increased serum levels and urinary excretion of octopamine in patients with PSE, but not in patients with coma from other causes, nor in cirrhotic patients without PSE: this group also showed a positive correlation between the degree of coma and the urinary excretion of octopamine. Manghani et al (1975) found the degree of PSE to be more closely related to serum levels than to urinary excretion of octopamine.

The synthesis of the evidence relating amino acid imbalance, false neurotransmitter formation and elevated levels of insulin and glucagon into a unifying hypothesis to explain PSE was proposed by Munro, Fernstrom and Wurtman in 1975. In essence, their proposal was that hyperinsulinaemia and portal-systemic

shunting in cirrhosis result in excessive degradation in the muscle of BCAAs. The reduced plasma concentration of BCAAs would result in reduced competition with TRP and other AAAs for entry into the brain, resulting in increased brain levels of TRP and 5-hydroxy tryptamine, which might also act as a false neurotransmitter amine. It was also noted shortly thereafter that increased levels of insulin could increase the transport of free TRP across the blood-brain barrier by a direct action (Daniel et al, 1975). Soeters and Fischer in the following year (1976) made the additional proposal that hyperglucagonaemia was disproportionately produced by PCS in dogs compared with hyperinsulinaemia, resulting in a relative "catabolic state" in which amino acids would be released in increasing concentration from endogenous stores. These groups of observations together constitute the amino acid/false neurotransmitter hypothesis in its complete and original form. Since the hypothesis was proposed in this form during the early progress of the work described in this Thesis, between the completion of the Glasgow studies and the commencement of the Cape Town work, discussion of the evidence for and against the validity of this hypothesis will be deferred until after the results of our own studies of amino acids, insulin and glucagon have been presented. It should be commented, however, that despite some problems in interpretation and application the work of Fischer and Munro and their colleagues presented at that time a very compelling case, and still to a large extent represents the most attractive single unifying hypothesis for PSE yet to be advanced.

1.8 Gamma-aminobutyric acid (GABA)

GABA is a metabolic product of glutamic acid, and as such may be formed as an end-product of the amination of α-ketoglutarate in the presence of excess ammonia (see Figure 5.7). The decarboxylation of glutamate to GABA is an irreversible reaction which has been shown to occur primarily in the neuronal mitochondria of grey matter. GABA is the principal inhibitory neurotransmitter of the mammalian brain: 25-45% of all nerve endings may be GABAergic. The brain content of GABA is 200- to 1000-fold greater than that of biogenic amine neurotransmitters such as dopamine, noradrenaline, acetylcholine and serotonin (Schafer and Jones, 1982). GABA may well be regarded as the current favourite in the search for a single chemical agent responsible for PSE (Lancet, 1984; Schafer and Jones, 1982). GABA causes neural inhibition by hyperpolarization of the post-synaptic neurone, and there is recent evidence indicating that the CNS actions of benzodiazepines and barbiturates are mediated by the GABA neurotransmitter system (see Baraldi et al, 1984 and Schafer and Jones for review of this literature). This observation forms an attractive clinical link since patients with decompensated liver disease are hypersensitive to these two classes of drugs.

Schafer and Jones and their co-workers have produced a number of pieces of evidence to support the role of GABA in PSE. Firstly, galactosamine-induced hepatic encephalopathy in rabbits was associated with the development of an abnormal pattern of visual evoked potentials which was similar to that of rabbits with benzodiazepine or barbiturate induced coma and

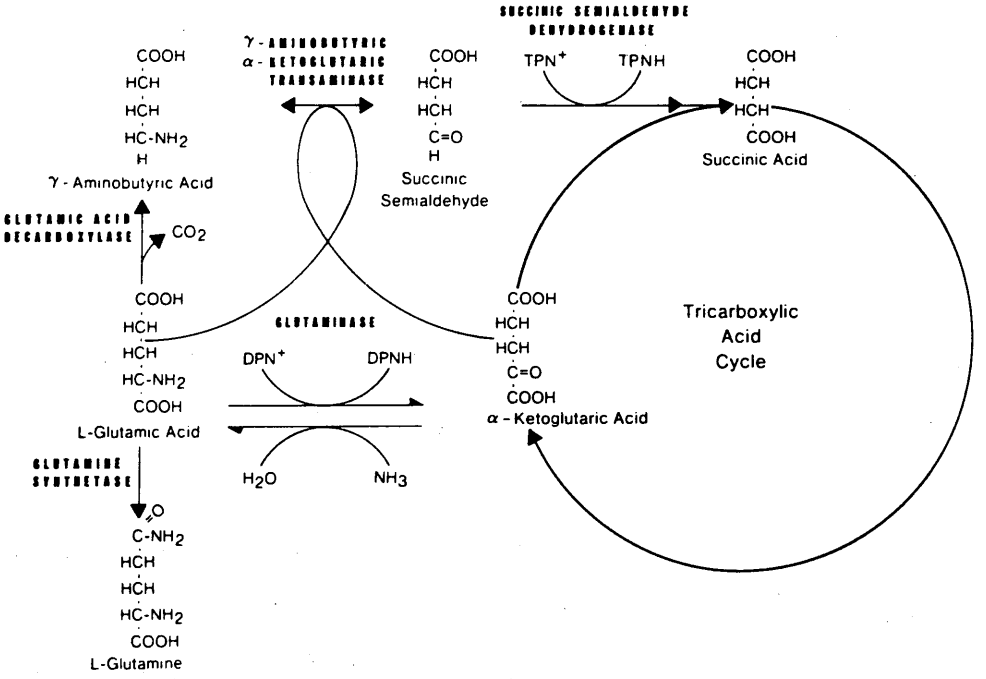


Figure 5.7

The metabolic relationships of GABA, ammonia, and the Krebs (TCA) cycle - the GABA Shunt.

encephalopathic rats, but fundamentally different from that obtained in rats with either ammonia or tryptophan induced coma. Secondly, high plasma levels of a GABA-like material have been found during encephalopathy in these rabbits. Thirdly, GABA production has been demonstrated in culture media on incubation with colonic bacteria, and portal levels of GABA in normal rabbits were found to be twice those of arterial plasma. Fourthly, GABA transaminase, the enzyme which catabolizes GABA, is found in high concentration in the liver, and hepatic failure is associated with decreased clearance of radiolabelled GABA from the plasma. Finally, elegant computerized imaging techniques were used to demonstrate increased blood to brain transport of radiolabelled γ-aminoisobutyric acid, a non-metabolizable isomer of GABA. This rise in permeability showed striking regional variation, greatest in certain grey matter areas of the brain, and was shown to occur some hours before the onset of overt hepatic encephalopathy.

These observations have been discussed at length here because they have post-dated the completion of the work undertaken for this Thesis, so that no studies of GABA were undertaken in this work. While the evidence cited above has been eloquently presented by these authors, it must be noted that the observations depend largely on a single rabbit model of acute hepatic encephalopathy, and may not be relevant to man. The same authors did show very high levels of GABA-like activity in the serum of patients with overt hepatic encephalopathy (Ferenci, Schafer et al, 1983), but other workers have found normal levels in several patients with PSE, as well as in the serum and brains

of rats with carbon tetrachloride induced liver disease (Goetcheus and Webster, 1965). Normal levels of GABA have also been reported in the brains of animals with advanced hepatic coma (Biebuyck et al, 1975; Schafer and Jones, 1982), although the latter authors have argued that minute changes in the total brain content of GABA may have a profound effect on neural activity. There is one report suggesting that GABA may actually exert a protective effect against ammonia intoxication in rats (Manning et al, 1964). Thus, while GABA is at present the rising star of PSE research, it remains to be seen whether it will burn out before reaching its zenith.

1.9 Acetylcholine

We have already noted that one possible action of ammonia in the CNS is inhibition of acetylcholine synthesis. However, Walker and his colleagues (1971) found normal acetylcholine levels in the cortex and brain stem in ammonia intoxicated animals. Later work from the same group produced data which was difficult to interpret, showing little effect on acetylcholine levels in animals with extremely high plasma ammonia, but depression of acetylcholine in those with mild hyperammonaemia. These studies and others are discussed in detail by Conn and Lieberthal (1979), but in essence the evidence for a major role for acetylcholine in PSE is scanty, and has not been further extensively investigated.

1.10 Phenols

Phenol levels have been shown to be increased in both liver failure and uraemia, and Zieve's group have proposed that these

compounds might form another component of the synergistic equation for the multifactorial production of PSE (Windus-Podehl et al, 1983). There does not appear to be any further confirmatory evidence of this hypothesis.

CHEMICAL CHANGES AFTER PORTAL DIVERSION - SELECTION OF STUDIES

At the commencement of this work on portal diversion, the published literature relating to the modern era of investigation into the pathogenesis of PSE was only just emerging. In particular, the Fischer-Munro hypothesis came to the fore after the completion of the Glasgow studies. Therefore, when the opportunity arose to carry out further studies in the University of Cape Town it was clear that plasma amino acid analysis should form a part of this further investigation. The time at which the Cape Town studies were performed saw a further rapid progression in the accumulating literature on the chemical changes in the central nervous system in PSE. Many of these advances, however, used complex chemical methods, with radioactive uptake studies and cell slice incubation techniques. The results of these studies will be discussed in the Chapter VI, but these techniques, although valuable, were not available to me at the time of this work.

Since there was evidence that some of the CNS changes might be a result of altered ammonia-glutamate-glutamine metabolism in the brain, it was decided that assays for these substances in brain tissue should be developed in order to examine these changes. Previous work using similar assays had been carried out in the University of Cape Town by Mans and Biebuyck and their

colleagues (Mans et al, 1979) but these studies were based upon assays performed on supra-tentorial brain tissue obtained by the freeze-blow technique. Because of the established distribution of histological changes and their concentration in areas of deep grey mattter, it was considered important to develop a technique for examining regional alterations in these substances, dividing the brain into cerebral cortex, cerebellum, and brain stem and mid-brain structures. These studies will be described in this section of the thesis.

2. EXPERIMENTAL DESIGN FOR BIOCHEMICAL STUDIES

2.1 Animals and groups

All of the experiments on plasma amino acids, amines and hormones, and on brain chemistry were performed exclusively at the University of Cape Town, and therefore Long-Evans rats were used for all of these studies. Estimation of chemical changes in the brain was not possible in the same animals as those from which histological brain material was to be obtained (Chapter IV). However, the broad design of the experiments included the same three groups described in Chapters III and IV above:

(a) Acute studies in animals bled at intervals and sacrificed at 72 hours according to the protocol described in Chapter III, Section 3.2. Material for both blood and brain chemistry was obtained in these animals.

(b) A chronic study in animals fed ad lib, housed four to a cage and weighed and bled at weekly intervals for 12 weeks according to the previously described protocol. In this group also both plasma and brain were available for biochemical studies.

(c) A pair-feeding study using the same animals as described in Chapters III and IV, pair-feeding control and PCT against PCS animals. Sacrifice was at 10 weeks in this study, and chemical studies were only possible on plasma, since the brains were fixed for the histological studies described in Chapter IV.

2.2 Laboratory methods

Sacrifice procedure

The animals were sacrificed at the appropriate time as described above, with laparotomy and cannulation of the aorta for blood sampling. Where brain biochemical studies were to be undertaken, the brain was not perfused at this point. Instead, the animal was turned to a prone position and the cranium rapidly exposed through a sagittal skin incision extending from the bridge of the nose to the cervical spine. The cervical muscles were cut transversely and the skin reflected to give a wide exposure of the posterior fossa of the skull. At this point, and before circulatory arrest took place, a deep transverse incision was made between the base of the skull and the topmost cervical vertebra, severing the spinal cord. The bone of the skull was then rapidly broken off from behind forwards by careful nibbling with an artery forceps. Once adequate exposure of the brain was obtained this could be removed with minimal trauma by sweeping a forceps around the lateral and anterior aspects of the brain, detaching the cranial nerves and olfactory lobes.

Division of the brain into cerebellum, cerebral hemispheres, and brain stem was then achieved along the lines of section shown in Figure 5.8. This dissection is based on the work of Glowinski and Iversen (1966), on whose studies Figure 5.8 is based. The dissection is carried out as follows:

The cerebellum (5) is first sectioned from the mid-brain and medulla oblongata/pons region (B-B).

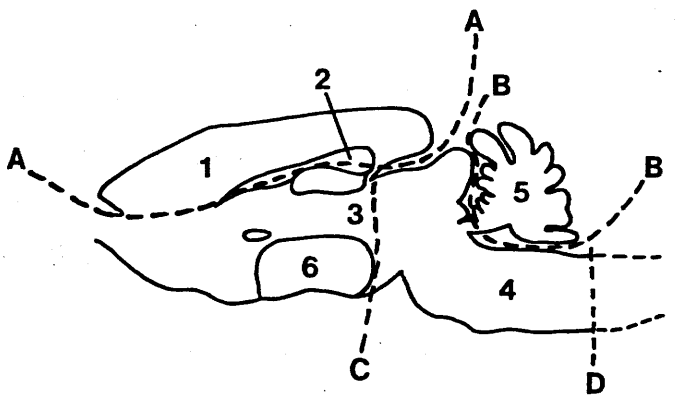


Figure 5.8

Diagram of sagittal section through rat brain, to show the regions into which brains were sectioned for the biochemical studies.

A-A, B-B, C and D represent the lines of section. The method is described in detail in the text.

The anatomical regions of the brain are identified numerically:

1. cerebral cortex
2. ventricle
3. mid brain
4. "brain stem"
   (thalamus, medulla oblongata, pons)
5. cerebellum
6. hypothalamus

(After Glowinski and Iversen, 1966)

The cerebral cortices are divided from the midline brain structures by retracting each cerebral hemisphere laterally from the midline, and dividing the corpus callosum to enter the lateral ventricle (2), and then dividing the structures uniting the cerebral cortex with the mid-brain in an almost vertical direction (A-A), in order to exclude any of the mid-brain structures (3). Thus each of the two specimens obtained contains exclusively cerebral cortical material. The small remnant of spinal cord is severed at the point at which it widens into the base of the brain (D). The remaining brain tissue is divided again just anterior to the mammillary bodies (C). (The tissue between these two lines of section (4) has been termed "brain stem" in these studies, although it contains a mixture of mid-brain, thalamus, subthalamic tissue, medulla oblongata, and pons. The reason for this plane of section was to ensure exclusion of the hypothalamus and pituitary (6).

Following the isolation of each of the brain regions described above, the fragment of tissue obtained was placed on a labelled piece of aluminium foil, which was dropped into liquid nitrogen. With practice it is possible to have all of the relevant brain tissue removed and frozen within one minute of sectioning the neck. Following this procedure the brain sections were transferred to storage flasks and kept in liquid nitrogen until further processing as described below.

Brain tissue extraction procedure

All tissues were extracted and assayed in appropriate batches in order to minimize problems due to inter-batch

variation of results.

The wrapped piece of brain tissue was removed from liquid nitrogen, and ground to a fine powder using a pre-chilled mortar and pestle under a constant layer of liquid nitrogen. As much as possible of the frozen brain powder was transferred to a pre-cooled, weighed plastic tube. This was then reweighed (weights of tissue extracted: cerebellum 100-300 mg, brainstem 80-250 mg, and cerebral cortex 250-970 mg). The tissue was then extracted with approximately six volumes of ice cold 8% perchloric acid, mixed well with a glass rod and allowed to stand for at least 10 minutes. The mixture was then centrifuged at 20000 G for 15 minutes at $4^{o}C$. The supernatant was then poured off and weighed in a pre-weighed cooled beaker. This was neutralized to pH 6-7 with 15% potassium hydroxide/15% potassium carbonate w/v, and the resultant neutral solution reweighed. This was then centrifuged at 20000 G for 10 minutes at $4^{o}C$, and the supernatant poured off for immediate assay or refrozen. All assays were done within five days of extraction, and the ammonia and tryptophan assays were carried out on the same day on unfrozen extract.

The extraction of blood for most of the assays was carried out in a similar way. A 2 ml blood sample was extracted with 2 ml of 8% perchloric acid. Neutralization and further treatment was as described for the brain extract.

Amino acid assays

These were performed on the extract of blood or brain as described above using a Beckmann amino acid analyser. All

results are exressed in µmol/g of frozen wet brain tissue, or in µmol/ml of plasma.

Glutamate and glutamine assay

This method was modified from that described by Lund (Bergmeyer "Methods of Enzymatic Analysis", 1974). Both assays are based on enzymatic conversion of L-glutamate to oxoglutarate (equation 2): for the glutamine assay L-glutamine is quantitatively converted to L-glutamate by incubation with glutaminase (equation 1).

(1) L-glutamine + H_2O $\xrightarrow{\text{glutaminase}}$ L-glutamate + NH_3

(2) L-glutamate + H_2O + NAD^+ $\xrightarrow{\text{glutamate dehydrogenase}}$ 2-oxoglutarate + NADH + NH_4^+

Enzymatic hydrolysis by glutaminase is specific for L-glutamine. Glutamate is therefore assayed in separate aliquots of the same sample, before and after glutaminase hydrolysis, and the difference between the measured glutamate content before and after hydrolysis represents the glutamine content. Details of the method may be found in Appendix A.

Tryptophan assay

The technique is based on that of Eccleston (1973). Tryptophan is condensed with formaldehyde and oxidized by ferric chloride in acid conditions to form a highly fluorescent substance called norharman, which is then measured fluorimetrically, and the concentration determined by comparison with known standards. Since tryptophan in the plasma exists partly free and partly bound to albumin, both free and bound

tryptophan were measured. Albumin binding is temperature dependent, so that all assays on plasma were carried out on fresh and not frozen samples. Free tryptophan is measured in an ultrafiltrate of fresh plasma, and total tryptophan measured in an unfiltered sample: bound tryptophan is the difference between these two readings. Only total tryptophan was measured in brain extracts. Further details of the method are included in Appendix A.

Ammonia assay

This was based on the method of Folbergrova et al (1969). The specimen is incubated with -ketoglutarate in the presence of glutamate dehydrogenase. In the presence of excess substrate and enzyme this is converted to glutamate and the reaction shift is measured spectrophotometrically and compared with known ammonium chloride standards. Great care must be taken to avoid contamination of reagents and glassware with ammonia-containing water, and deionized water was used throughout these studies. The method is described in detail in Appendix A.

Hormone assays

An aliquot of each plasma sample to which aprotinin powder had been added was used for measurement of insulin and glucagon. This was performed by radioimmunoassay as described by Botha et al (1977).

3. RESULTS

3.1 Plasma amino acids

Tables 11-18 in Appendix B show all of the amino acid data from the animals studied. The amino acids shown here are valine (VAL), isoleucine (ILEU), leucine (LEU), tyrosine (TYR), phenylalanine (PHE), glycine (GLY), alanine (ALA) and methionine (METH). The amino acids which were of particular interest were the branched chain molecules (VAL, ILEU and LEU) and the aromatic group (TYR and PHE). The means of these values are therefore shown in Tables 5.2 and 5.3, and graphically in Figures 5.9-5.11. In addition, the ratio of the total of the branched chain (VAL + ILEU + LEU) to the total aromatic (TYR + PHE) amino acids is shown in the Tables and in Figure 5.12. Tables 5.2 and 5.3 also indicate levels of statistical significance (by Student's t test for unpaired data) amongst the groups in each experiment, between values obtained at 72 hours and at 10 weeks after operation in ad lib fed animals, and between ad lib and pair-fed animals at 10 weeks.

(a) Acute experiment (Figure 5.9)

In this experiment the only significant difference between control and PCT animals was a 20% reduction in the plasma LEU level. However, when compared with control animals PCS rats showed a significant reduction in VAL and LEU, and significant elevations in TYR and PHE at 72 hours. There were no significant differences in levels of the branched chain amino acids between the PCS and PCT animals, but TYR and PHE were significantly

TABLE 5.2

Plasma amino acids (μmol/ml) 72 hours after operation in control, PCT and PCS rats. Values shown are mean ± 1 SEm. (Data in full in Appendix B Tables 11-13).

	VAL	ILEU	LEU	TYR	PHE	V+I+L / T+P
CONTROL (11)	142 ±18	51 ±5	120 ±10	64 ±3	69 ±10	2.35 ±0.25
PCT (11)	105 ±8	45 ±4	90 ±8	71 ±5	69 ±4	1.70 ±0.08
PCS (12)	87 ±6	43 ±3	73 ±7	107 ±7	97 ±6	1.00 ±0.06

P values (Student's t-test: values > 0.05 not shown)

	VAL	ILEU	LEU	TYR	PHE	V+I+L / T+P
C vs PCT	---	---	<0.05	---	---	---
C vs PCS	<0.01	---	<0.001	<<0.001	<0.05	<<0.001
PCS vs PCT	---	---	---	<0.001	<0.001	<<0.001

72 hrs vs 10 weeks (Table 5.3)

	VAL	ILEU	LEU	TYR	PHE	V+I+L / T+P
CONTROL	---	<0.005	---	---	---	<0.05
PCT	---	<0.05	<0.05	---	---	<0.01
PCS	---	---	---	---	---	<0.05

TABLE 5.3

Amino acids (μmol/ml) 10 weeks after operation in (a) ad lib fed and (b) pair-fed. Values shown are mean ± 1 SE_m. (Data in full in Appendix B, Tables 14-17).

(a) AD LIB	VAL	ILEU	LEU	TYR	PHE	V+I+L / T+P
CONTROL (14)	164 ±15	75 ±6	145 ±12	61 ±4	60 ±5	3.14 ±0.13
PCT (9)	135 ±19	63 ±7	137 ±17	68 ±6	77 ±6	2.30 ±0.19
PCS (9)	97 ±6	39 ±2	83 ±8	94 ±4	91 ±5	1.20 ±0.07
(b) PAIR-FED						
CONTROL (3)	84 ±4	54 ±6	97 ±13	35 ±6	51 ±9	2.86 ±0.35
PCT (5)	62 ±9	42 ±1	72 ±6	42 ±6	58 ±4	1.97 ±0.20
PCS (6)	72 ±10	43 ±3	83 ±6	74 ±7	73 ±9	1.40 ±0.57

P values (Student's t-test: values > 0.05 not shown)

AD LIB	VAL	ILEU	LEU	TYR	PHE	V+I+L / T+P
C vs PCT	---	---	---	---	<0.05	<0.01
C vs PCS	<0.01	<0.001	<0.01	<0.001	<0.001	<0.001
PCS vs PCT	---	<0.01	<0.01	<0.01	---	<0.001
PAIR-FED						
C vs PCT	---	---	---	---	---	---
C vs PCS	---	---	---	<0.001	<0.01	---
PCS vs PCT	---	---	---	<0.01	---	---
AD LIB vs PAIR-FED						
CONTROL	<0.001	<0.05	<0.02	<0.01	---	---
PCT	<0.01	<0.05	<0.01	<0.01	<0.05	---
PCS	---	---	---	<0.05	---	---

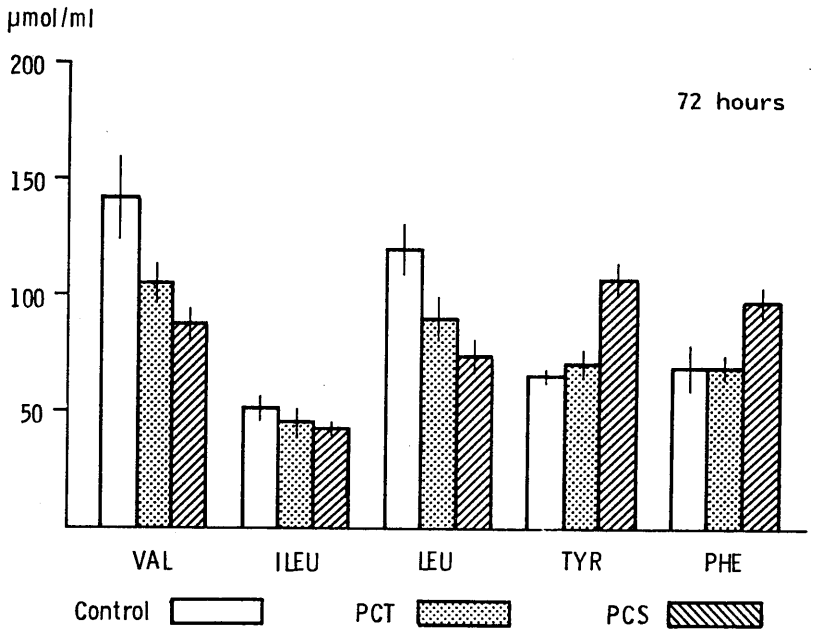


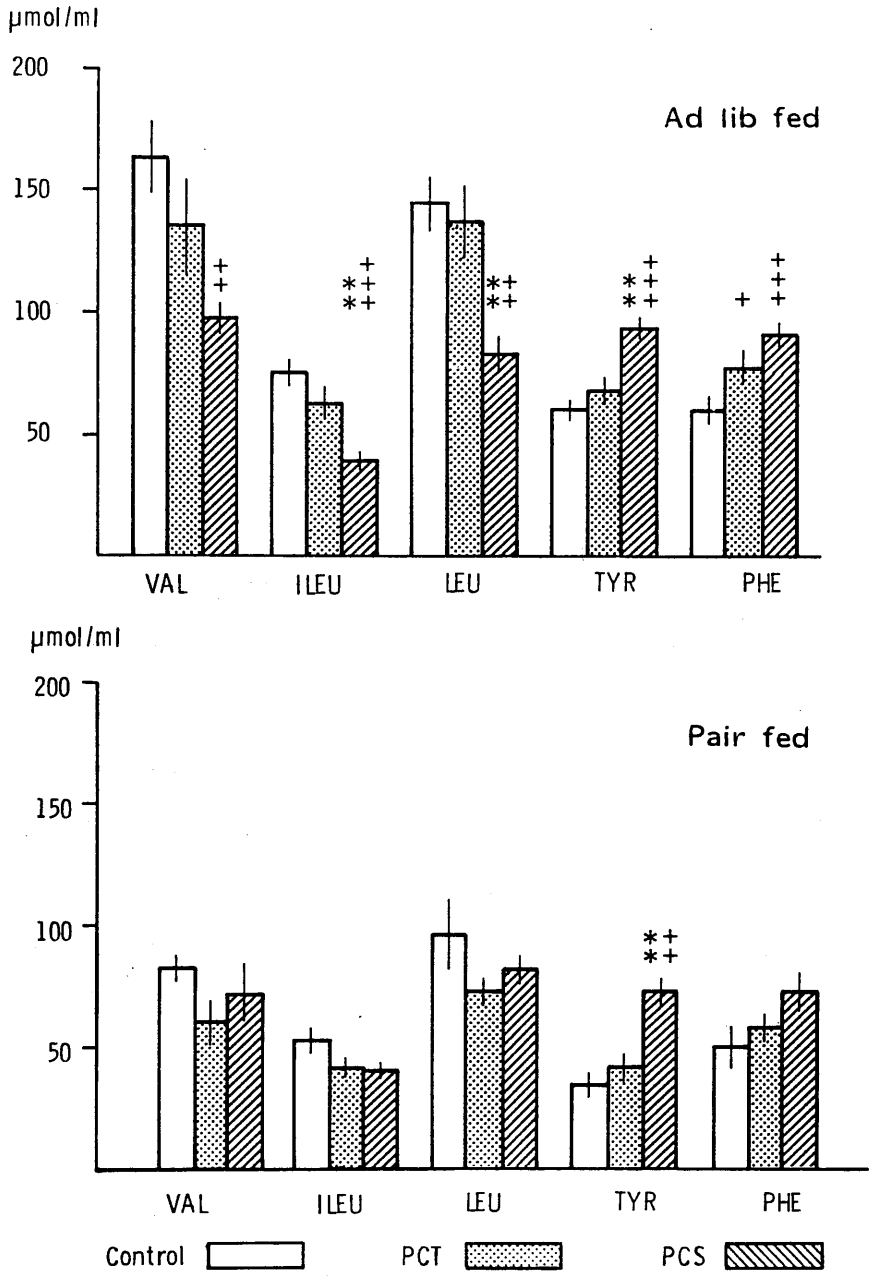
Figure 5.9

Plasma amino acids (µmol/ml) 72 hours after operation in ad lib fed rats.

Each block of three vertical bars represents the mean values for a single amino acid in each of the three experimental preparations in the order Control, PCT, PCS. Values are shown as means and standard errors of the mean.

The amino acids shown in this and in the next two figures are:

VAL valine ILEU isoleucine LEU leucine
TYR tyrosine PHE phenylananine



Figures 5.10 and 5.11

Plasma amino acids (µmol/ml) 10 weeks after operation. The upper figure (5.10) shows values for ad lib fed rats, and the lower (5.11) for pair-fed rats.

The layout of results is exactly as described for the previous figure (5.9).

Statistically significant comparisons amongst groups:

P vs Control		P vs PCT	
+	<0.05	*	<0.05
++	<0.01	**	<0.01
+++	<0.001	***	<0.001

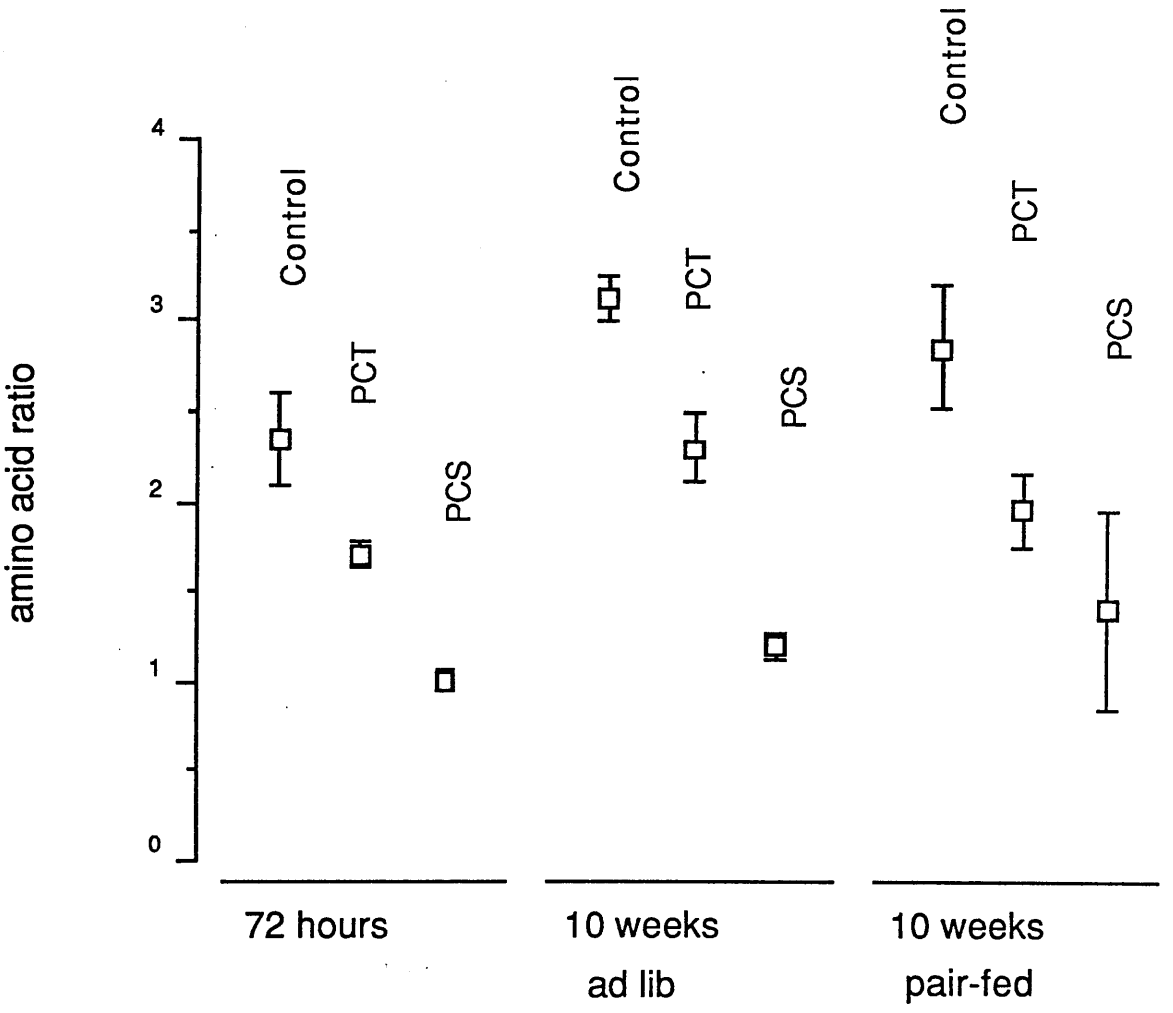


Fig. 5.12 Amino acid ratio (VAL+LEU+ILEU)/ (TYR+PHE) in plasma 72 hours and 10 weeks after operation in Control, PCT and PCS rats.
All values shown as means and standard errors of the mean.

higher after PCS than after PCT.

The ratios of branched chain to aromatic amino acids at 72 hours and 10 weeks are shown in Figure 5.12. There was no significant difference between control and PCT animals at this time, but there was a highly significant reduction in the ratio for PCS rats compared with both control and PCT.

(b) Chronic experiment with ad lib feeding (Fig.5.10)

The mean levels of all three branched chain amino acids was greater in control and PCT animals than those observed at 72 hours, though this only achieved statistical significance for ILEU (both control and PCT) and for LEU (PCT only). The ratio of branched chain to aromatic amino acids was significantly higher in all three groups at 10 weeks than at 72 hours (Figure 5.12).

At ten weeks in the PCS animals the mean levels of branched chain amino acids were lower than those after control or PCT, and those of the aromatic amino acids higher. These differences reached a high level of statistical significance when compared with controls for all the amino acids, but the differences between PCS and PCT in VAL and PHE failed to achieve statistical significance. The only statistically significant difference between control and PCT animals was an increase in PHE.

There was a statistically significant reduction in the branched chain to aromatic amino acid ratio following PCT, but an even more marked reduction in PCS animals, which differed significantly also from PCT at this point (Figure 5.12).

(c) Chronic experiment with pair-feeding (Fig.5.11)

In the control and PCT groups there was an overall reduction in the mean levels of all of the amino acids compared with ad lib fed animals. This difference was statistically significant in all cases except for the reduction of PHE in the control group. In comparing ad lib and pair-fed PCS animals, the only change was a reduction in plasma TYR. There was a fall in the amino acid ratio in both control and PCT groups, but these changes were not statistically significant.

Comparing experimental groups in the pair-feeding experiment, there were now no significant differences in the branched chain amino acids amongst the three groups. However, the levels of TYR and PHE remained higher in the PCS animals than either PCT or control, and the differences were statistically significant for both TYR and PHE compared with control animals, and for TYR compared with PCT. The difference in PHE between PCS and PCT was not statistically significant.

3.2 Plasma hormone levels (Fig.5.13)

Levels of insulin and glucagon were available for study in a smaller number of animals from the chronic ad lib feeding study only (control 6, PCT 6, PCS 8). The individual values are shown in Table 5.4. There was a significant increase in the level of plasma insulin in both PCT and PCS animals compared with control. Levels of plasma glucagon were similarly elevated. However, the molar ratio of insulin to glucagon did not differ significantly amongst the groups, indicating an equivalent degree

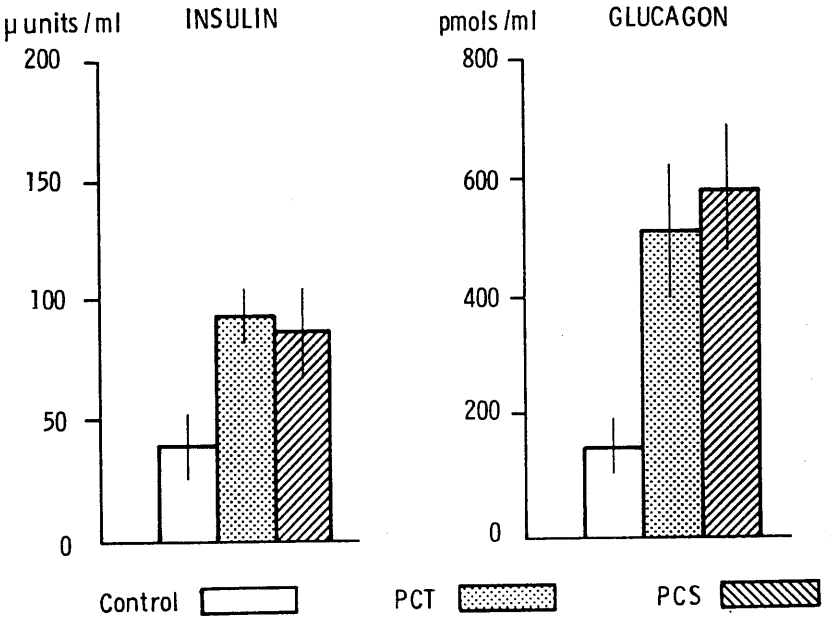


Figure 5.13

Plasma insulin (μ-units/ml) and plasma glucagon (pmols/ml) 10 weeks after operation in ad lib fed rats.

Values are shown as means and standard errors of the means.

TABLE 5.4

Plasma insulin and glucagon 10 weeks after operation in ad lib fed rats.

	INSULIN (μU/ml)	GLUCAGON (pg/ml)	MOLAR RATIO I:G
CONTROL	29	167	3.43
	11	37	8.01
	107	---	----
	16	283	1.52
	37	80	12.46
	41	191	5.78
Mean ± SEm	40 ± 14	152 ± 43	6.24 ± 1.9
PCT	67	401	4.50
	86	719	3.22
	76	956	2.14
	76	337	6.07
	115	214	14.47
	145	459	8.51
Mean ± SEm	94 ± 12	514 ± 112	6.49 ± 1.8
(p vs Control	<0.01	<0.01	>0.05)
PCS	91		
	30	259	3.07
	149	378	10.62
	106	974	2.93
	20	820	0.66
	30	487	1.66
	129	419	8.29
	145	740	5.28
Mean ± SEm	87 ± 19	528 ± 100	4.64 ± 1.38
(p vs Control	<0.05	<0.005	>0.05)

of elevation of both hormones in each of the two experimental groups.

3.3 Ammonia and amine changes

(a) Ammonia

The results for plasma ammonia estimations are shown in Figure 5.14, and the detailed data are in Appendix B, Table 19. The results shown are for animals which were fed ad lib and sacrificed at ten weeks. Plasma ammonia levels were elevated to the same degree following PCT and PCS compared with control animals.

Brain ammonia levels in the cerebral cortex, brain stem and cerebellum are shown in Figure 5.15. Levels of ammonia in the cerebral cortex were low in all three groups, with no significant differences. In the brain stem and cerebellum, ammonia levels were almost twice those found in the cerebral cortex in control and PCT animals, although this change just failed to achieve statistical significance for cerebellar ammonia in the PCT group. Ammonia levels did not differ between control and PCT animals in either the brain stem or cerebellum. In these two regions, however, there was marked elevation of ammonia following PCS, with significant increases over both control and PCT values.

Figure 5.14 shows the plasma ammonia levels in the pair-fed animals. As in the ad lib fed groups, both PCT and PCS show significant elevations compared with control, but in this series

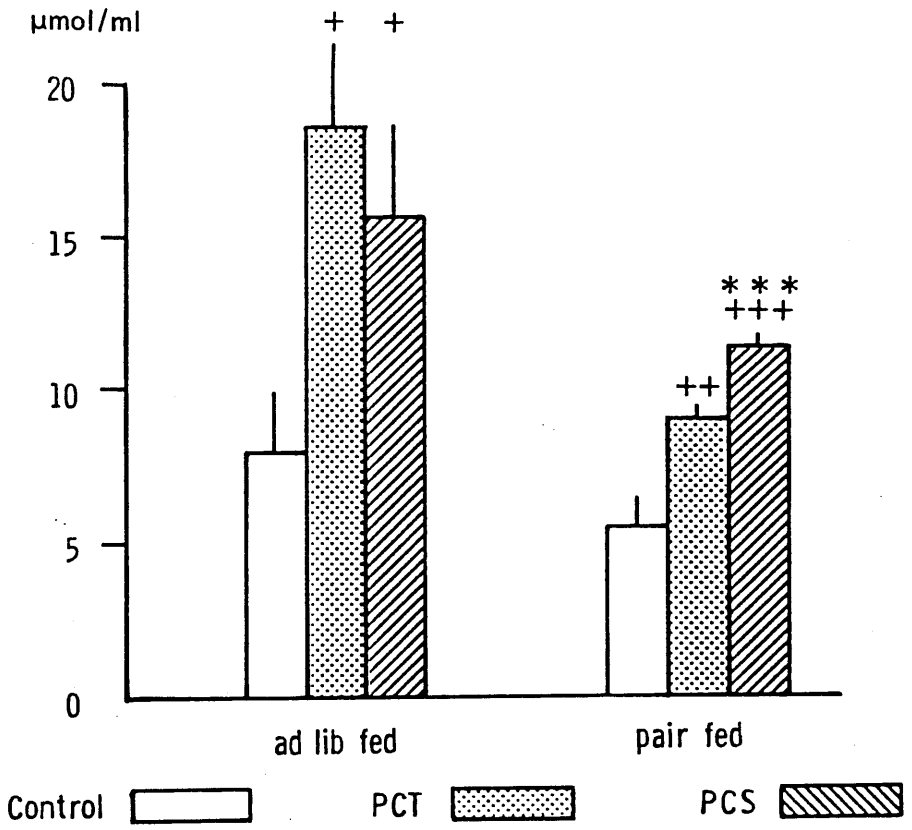


Figure 5.14

Plasma ammonia (µmol/ml) 10 weeks after operation in ad lib fed (left) and pair fed (right) rats.

Statistically significant differences amongst groups:

P vs Control	+	<0.05	P vs PCT	*	<0.05
	++	<0.01		**	<0.01
	+++	<0.001		***	<0.001

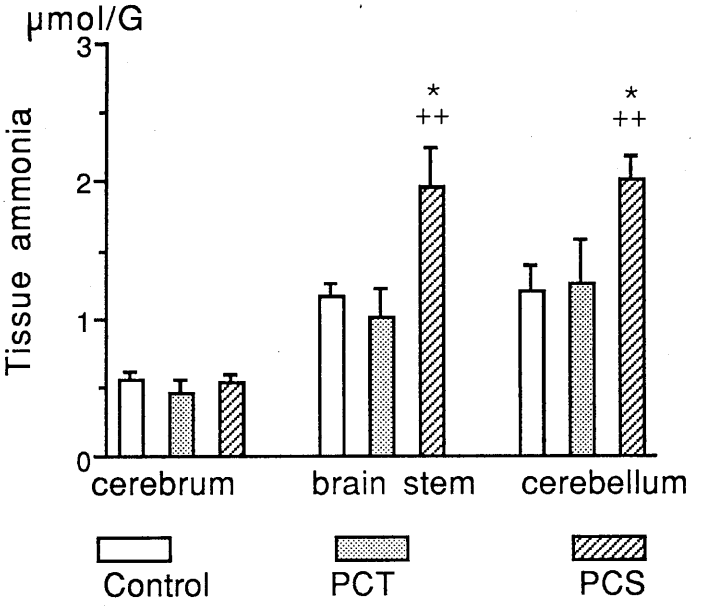


Figure 5.15

Ammonia levels (µmol/G) in three brain regions 10 weeks after operation in ad lib fed rats.

Each bar represents the mean of one group, and standard errors of the means.

Statistically significant differences:
P vs Control ++ <0.001
P vs PCT * <0.05

levels were significantly higher after PCS than after PCT. The levels of ammonia in the three brain regions studied is shown alongside blood levels for the pair-fed animals in Appendix B, Table 19. There were a number of surprisingly high levels recorded in all groups in this series, and this wide range of values, with the availability of only two animals in the pair-fed control group, make statistical comparison amongst the groups unhelpful. However, it can be seen in Appendix B Table 20 that the finding of higher levels in the brain stem and cerebellum seen in the ad lib fed group was observed in these animals also, although there are no apparent differences amongst the experimental groups in the pair-fed series.

Plasma and brain ammonia levels in the acute experiment (72 hours after operation) are shown in Appendix B, Table 21. This group contains too few animals for statistical comparison.

(b) Glutamate, glutamine and tryptophan.

The values for glutamate, glutamine and tryptophan in the brains of animals fed ad lib and sacrificed ten weeks after operation are shown in Appendix B, Tables 22 and 23. The results are shown graphically for glutamate and glutamine in Figures 5.16 and 5.17.

Although values of glutamine in the brain stem and cerebellum in PCS animals tended to be higher than those seen in controls, there are in fact no statistically significant differences amongst these groups. Similarly, the somewhat lower levels of glutamate in the cerebrum and in the cerebellum just failed to reach a statistically significant degree of reduction.

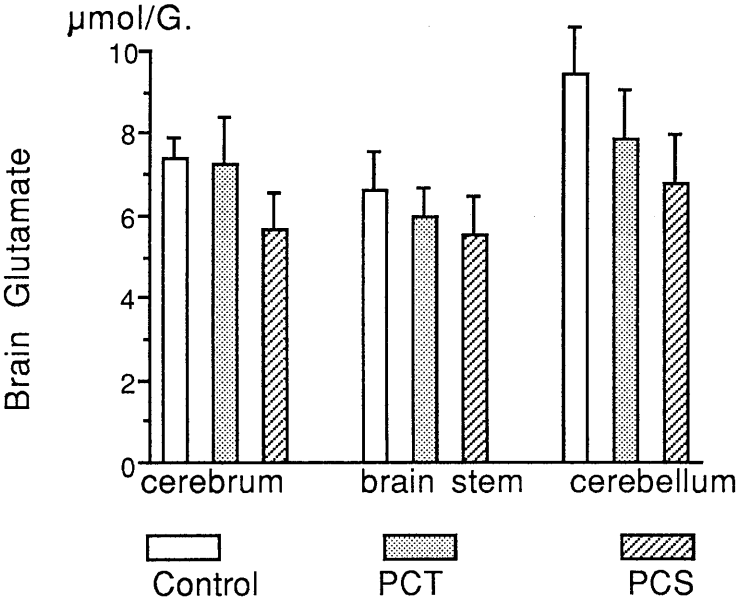


Fig. 5.16 Brain Glutamate concentrations (µmol/G wet weight) 10 weeks after operation in ad lib fed rats. Values are shown as means and standard errors of the means.

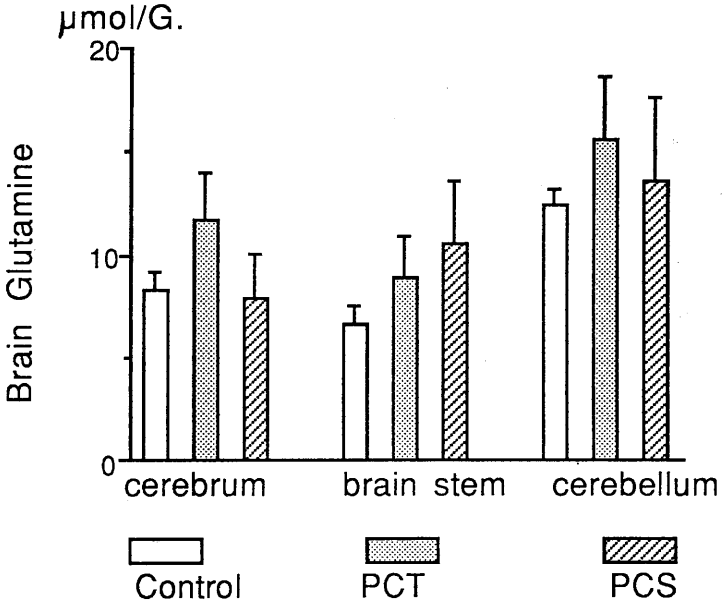


Fig. 5.17 Brain Glutamine levels 10 weeks after operation in ad lib fed rats.
Values shown are means and standard errors of the means.

Values for tryptophan in the brain 10 weeks after operation showed a wide variation. Levels were higher in brain stem and cerebellum than in the cerebral cortex in all groups, but there were no intergroup differences.

(c) Other amino acids

Amino acid levels in the brain were available from the pair-feeding studies only. These results are shown graphically in Figure 5.18, and the data are included in Appendix B, Tables 24-26. It can be seen that there was wide variability in the amino acid results, so that no clear pattern was discernible. Extremely high levels of PHE and TYR were recorded in only one animal, belonging to the PCS group.

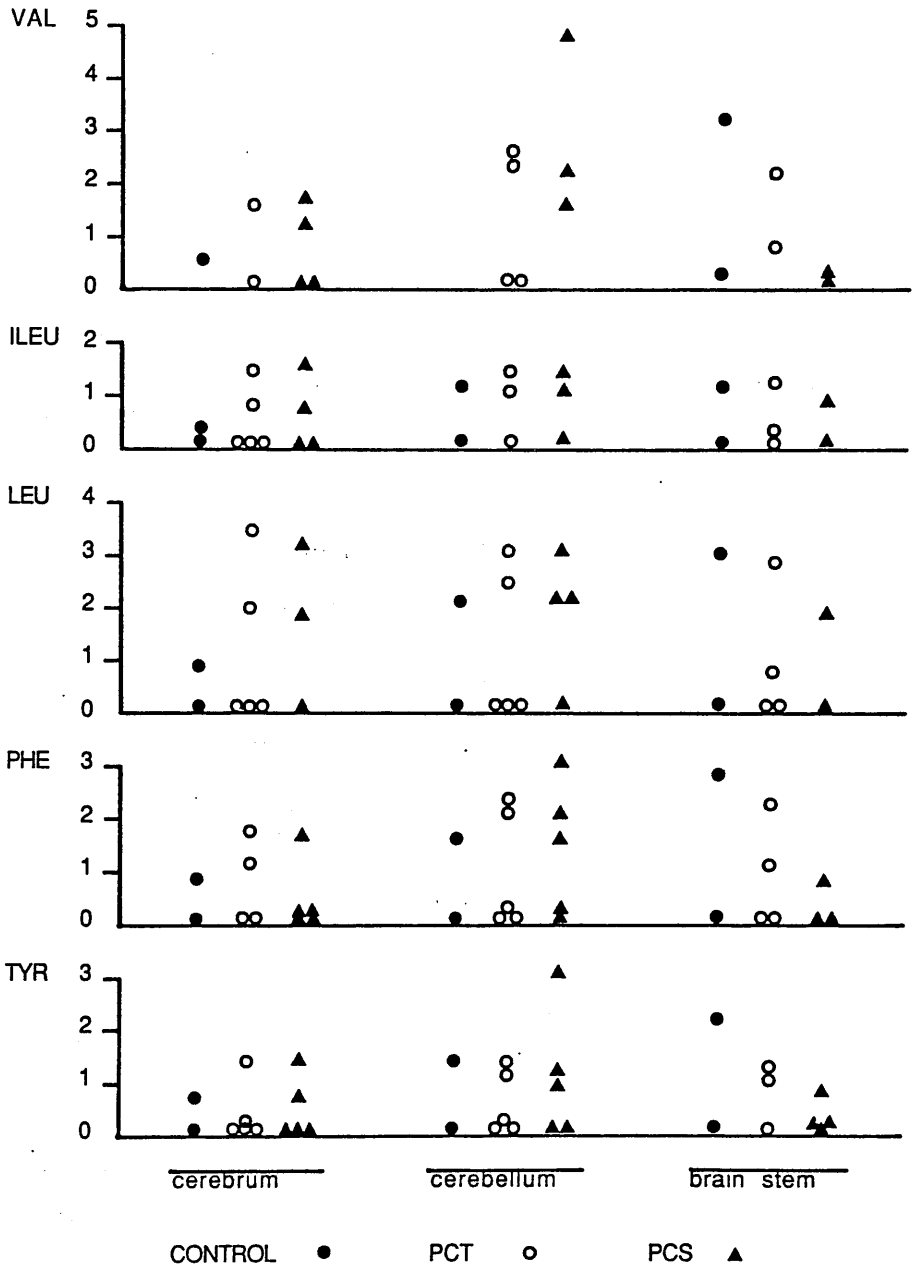


Figure 5.18

Tissue amino acid levels (μmol/G) in three brain regions 10 weeks after operation in pair-fed rats. Each point shown is the value in a single sample from one rat.

4. BIOCHEMICAL CHANGES AFTER PCS AND PCT - DISCUSSION

4.1 Amino acids and hormones

The present results accord with the pattern of amino acid changes seen by other workers following PCS and that seen in liver disease in man (Fischer et al, 1974; Rosen et al, 1977; Aguirre et al, 1974; Walshe, 1953; Iber et al, 1957). The pattern is essentially one of reduction in the BCAAs and elevation of the AAAs. Differences between PCS and PCT had become evident by 72 hours in the acute experiment. PCS rats already already show significant elevations in TYR and PHE at this point, which may be an early indication of hepatocellular metabolic derangement related to deprivation of inflow into the portal tracts. PCT animals showed no such abnormalities. Fischer's ratio (VAL + ILEU + LEU to TYR + PHE) never fell below 1.0 in the control and PCT groups at 72 hours, but was less than 1.0 in 7 out of 12 rats in the PCS group. All groups had reduced levels of BCAAs at 72 hours, resulting in rather low ratios even for the PCT and control animals: this presumably is an acute effect of operative trauma.

By 10 weeks the reduced BCAA levels had returned to normal in the control and PCT groups but remained at the depressed level in the PCS animals. This finding in PCS animals is the same as that reported by Fischer and his colleagues (Soeters and Fischer, 1976; Soeters et al, 1977), and is attributed to increased catabolism of BCAAs in muscle and in adipose tissue. These workers suggested that a shift to a catabolic state was largely responsible for this disturbance in peripheral amino acid

balance, and they showed in dogs that this was related to hyperglucagonaemia in excess of hyperinsulinaemia. However, the present results in ad lib fed rats demonstrated proportionate elevations of insulin and of glucagon, so that it is difficult to postulate an insulin-glucagon imbalance in this series of experiments. Moreover, the insulin to glucagon ratio remained the same even in PCT animals, and the hormones showed the same degree of elevation as that observed in PCS rats.

We have already noted that pair-feeding produced a degree of weight loss and retardation of subsequent growth in control and PCT animals similar to that regularly seen after PCS (compare Figures 3.1 and 3.21). This was reflected in the significant overall reduction in the levels of all amino acids in pair-fed control and PCT animals compared with those fed ad lib. The amino acid levels observed in the PCS animals in the pair-feeding experiment remained in the same range as those in PCS animals fed ad lib: this result is useful confirmation of the comparability of the PCS animals in the two experiments.

It has been noted that elevation of TYR and PHE was evident in PCS animals at 72 hours, and this persisted to ten weeks in the ad lib feeding experiment. At this time there was marked elevation of TYR and PHE in the PCS rats compared with both control and PCT. It is interesting to note that a pattern begins to emerge with the amino acid results which persists throughout all of the other biochemical studies, with a stepwise progression of values from control to PCT to PCS in a consistent direction for each group of animals. Figures 5.10 and 5.11 show which of these changes reaches statistical significance, and in the case

of the amino acid profile there are significant differences in the majority of cases between PCS and PCT as well as between PCS and control.

The levelling of BCAA changes amongst the three groups as a result of pair-feeding, while the differences in AAAs persisted, is in keeping with the concept that the BCAAs are largely determined by nutritional factors, while AAA levels depend upon intact hepatic metabolism. So far our results are in keeping with the amino acid hypothesis of Fischer and his colleagues, and further demonstrate the importance of both portal-systemic shunting and hepatocellular atrophy and dysfunction in causing or permitting the development of PSE. These results must be viewed in combination with the markedly greater incidence of histological changes in the CNS after PCS than after PCT. It must also be noted that in the small group of animals available for histological studies in the pair-feeding experiment, this difference in structural CNS damage persisted.

Hyperinsulinaemia in chronic liver disease is thought to be largely due to diminished insulin degradation, and as such is greatest in patients with impaired liver function (Smith-Laing et al, 1979). We have already noted that in these studies insulin and glucagon levels were increased to the same extent following PCS and PCT, so that if hepatic function is preserved with regard to hormonal degradation in the PCT preparation, we may conclude that these elevated hormone levels have been determined principally by portal-systemic diversion, rather than by hepatocellular dysfunction. The number of animals in which hormone changes were studied is limited, and unfortunately

hormone studies were not available from the pair-feeding experiment. Moreover, these studies may not be directly comparable with the clinical situation. In patients with chronic liver disease, further changes in the ratio of glucagon to insulin occur with the onset of hepatic decompensation, and the present model may relate more closely to patients with chronic well-compensated liver disease. There is clinical evidence that in patients with cirrhosis, the degree of hyperglucagonaemia increases after portal-systemic shunting, and may be determined by the size of the shunt rather than by hepatic dysfunction (Dudley et al, 1979).

4.2 Plasma and brain ammonia

Ammonia levels were markedly and significantly elevated over control values in both PCS and PCT animals in the *ad lib* fed study (Figure 5.14). However, there was no difference in the degree of elevation between PCS and PCT animals. While this is in contrast with other workers who have shown decreased ammonia tolerance following PCS, but a lesser degree of ammonia intolerance following PCT (Kirsh et al,1964; Turney et al, 1966) the discrepancy may not be a severe one. These animals were fed *ad lib* on normal laboratory chow with a protein content of 15.9g/100g, and no attempt was made to stress load the animals with ammonia. Moreover we have noted that the PCS animals were anorexic, and therefore consuming a smaller nitrogen load than their PCT counterparts. It may be that a reduced ammonia tolerance would be detected in the PCS animals as compared with PCT had this been studied. However, it should also be noted that impaired ammonia tolerance may be the most reliable indicator of

patency in portacaval anastomoses (Orloff et al, 1963), so that at these levels we may simply be observing evidence of effective portal-systemic diversion, with no specific effect of impaired hepatic function.

By contrast, there were marked differences in the ammonia levels in brain tissue (Figure 5.15). Levels of ammonia in the cerebral cortex were low in all groups of animals, but were higher (though not statistically so) in brain stem and cerebellum for control and PCT animals. Elevation of ammonia levels in these two regions following PCS was significantly higher than control or PCT values. It is interesting that it is in these regions that histological changes are at their most marked, and it may be important to note that this pattern of changes was observed for other substances studied in the brain in these experiments. It may be suggested that it is in these areas that both metabolic and structural abnormalities make their mark, containing as they do the regions which relate functionally to the syndromes of PSE.

The disproportionate elevation of ammonia in the brain stem and cerebellum in PCS animals compared with PCT is interesting in the presence of equally elevated plasma ammonia levels in both groups. Thus the elevation of ammonia in brain does not simply reflect increased diffusion from the plasma, but may relate either to increased metabolic production or decreased ammonia trapping, or to differences in blood-brain barrier permeability. Either of these views may be consistent with theories of the pathogenesis of PSE, and this will be discussed in the final chapter of this Thesis.

A similar pattern to that described was observed in the pair-fed animals, although in this case plasma ammonia levels were significantly higher after PCS than after PCT. It is not clear (Appendix B, Table 20) why there was such a wide range of values observed in this group of animals, but this unfortunately renders useful comparison impossible for this phase of the study.

4.3 Other brain chemistry

The consistent pattern previously referred to relating control, PCT and PCS animals can be seen in the results for glutamate and glutamine (Figs. 5.16, 5.17). Reduction of glutamate in the cerebrum and cerebellum following PCS just failed to reach a statistically significant level ($p<0.05$ on single-tailed testing by Student's t statistic), but no other significant differences were seen. However, the trends in these values remain interesting. It has been demonstrated (Berl et al, 1969; Berl and Clark, 1975) that there is important compartmentation of glutamine and glutamate metabolism in the CNS. There appear to be at least two distinct pools, a large slow turnover pool and a much smaller rapid turnover pool. It is likely that the latter is related to neurotransmission, with the release of glutamate at the synapses and its re-uptake after condensation with ammonia to glutamine into the glial cells, there to be reprocessed to glutamate via α-ketoglutarate (see Figure 5.2). The differential size of these pools will result in masking of quantitatively small but highly important changes in the metabolically active small pool. Thus, while one cannot attach true significance to the changes observed in this study

examination of small trends may be of relevance in considering the hypotheses relating to chemical changes within the brain. The possible importance of these relationships will be discussed in Chapter VI.

Unfortunately, the results of plasma and brain tryptophan estimation suffer from a wide spread of values, and do not allow any valid conclusions to be drawn.

Hypotheses regarding the possible neurochemical significance of the present findings can only be very tentatively made. In the next section these findings will be discussed in relation to our other results, and also in relation to work from other groups on the possible neural mechanisms of PSE. In particular we will examine the role of glutamine and glutamate in relation to ammonia metabolism and glial cell function.

CHAPTER VI. GENERAL DISCUSSION

1. PSE IN RELATION TO LIVER BLOOD FLOW, MASS AND FUNCTION

There is now considerable evidence, much of it reviewed elsewhere in this thesis, that maintenance of liver function provides protection against PSE in the shunted animal. The reason why this should be so remains uncertain, but the process is probably multifactorial, in keeping with the extremely varied metabolic functions of the intact liver. The observation that maintenance of hepatic blood flow protects liver function is much less clear-cut, and takes us into an area of research extending back some 20 years attempting to distinguish effects related to quality of flow from those related to quantity. This distinction will be discussed in Section 2 of this Chapter. In this Section we will examine the clinical and experimental evidence relating hepatic blood flow, mass and function to the occurrence of PSE.

1.1 PSE is increased by surgical shunting

The clinical evidence for a relationship between PSE and shunting is fraught by the variable susceptibility to the development of PSE in different forms of liver disease, the occurrence of both spontaneous (pathological) and iatrogenic (surgical) portal-systemic shunts, and the paucity of studies of the true epidemiology of PSE. Furthermore, the majority of clinical studies published fail to define PSE or "severe" PSE carefully: retrospective studies are uniformly inadequate in identifying PSE accurately. Amongst the most reliable figures

are those arising from the West Haven-Connecticut prospective studies of prophylactic portacaval anastomosis carried out by the Boston Interhospital Liver Group (Mutchnick et al, 1974; Conn et al, 1972). These workers recorded neurological changes prospectively and defined mild PSE as three or fewer episodes of encephalopathy precipitated by gastrointestinal bleeding, diuretic or depressive drugs, or by terminal events. Severe PSE was defined as more than three episodes not induced by gastrointestinal bleeding or drugs, or continuous symptoms, or a requirement for prolonged protein restriction or oral antibiotic therapy. All patients were cirrhotic, and mostly alcoholics, and were randomized into an observation group and a prophylactic end-to-side PCS group. Almost 20% of both groups had experienced at least a single episode of PSE before inclusion in the trial, but only one patient had suffered severe spontaneous recurrent PSE (in the control group). During a mean period of follow-up of four years after inclusion, the incidence of PSE was studied in those patients who had not previously experienced the syndrome (49 control and 32 PCS). Mild individual episodes of PSE occurred in approximately one third of each group, with no statistically significant difference. However, severe recurrent PSE occurred in some 20% of patients with prophylactic shunts, but in only 2% of the unshunted control patients, a highly significant statistical difference. This careful study demonstrated that although cirrhotic patients with or without PCS may develop sporadic individual episodes of mild PSE, the severe continuous and incapacitating form of PSE occurs almost exclusively in patients with shunts. It should be noted parenthetically that this study completed in 1969 (Resnick et al,

1969) also failed to demonstrate improved survival in shunted patients, and may be said to mark the close of the era of unbridled enthusiasm for shunting in cirrhosis.

The same group reported the results of a similar controlled study for the therapeutic portacaval shunt in 1974 (Resnick et al, 1974). In this study there was no substantial difference in the incidence of acute hepatic encephalopathy between the PCS group (24%) and the medically treated group (17%), although the incidence of acute encephalopathy was higher at 32% following end-to-side PCS than side-to-side (14%) - not a statistically significant difference. By the same definitions as in the previous study, there was no statistically significant difference in chronic PSE between the groups, although the actual incidence was higher for medically treated patients (8/23 = 35%) than PCS patients (9/46 = 20%). However, this apparently higher rate amongst unoperated patients was due entirely to retention within this group of 7 patients who required an emergency end-to-side PCS during the course of the study because of variceal haemorrhage: 3 of these patients developed PSE. Moreover, the randomly selected medical patients had at the onset of the study a higher frequency of prior PSE (57% vs 34%). Thus in this study there are a number of factors which frustrate any attempt to demonstrate biologically significant differences between the groups. In comparing severe PSE in this study the incidence in the medical group was only 4% (1/23) compared with 13% (6/46) in the surgical group: the authors do not comment on this result, but in fact statistical testing does not reveal any significant difference.

Similar data have been reported from other controlled studies. Rueff and his colleagues (1976) reported a controlled study in 89 alcoholic cirrhotics randomized between 1968 and 1971 to end-to-side PCS or to medical treatment. Once again, as in the Boston Inter-hospital Liver Group study, the incidence of episodes of acute encephalopathy did not differ between the groups (PCS 10/24, control 10/36). However, no incidence of chronic PSE was seen in the control group, while 6/24 patients developed this syndrome after PCS (25%). More recently, Reynolds and his colleagues (1981) reported from Los Angeles a seven year study of 89 patients with alcoholic liver disease and variceal bleeding entered into a randomized controlled trial of medical therapy vs end-to-side PCS. All surviving patients had at least five years of observation. Once again the incidence of hepatic encephalopathy related to gastrointestinal bleeding or during periods of hepatic decompensation did not differ between the two groups, but the development of spontaneous PSE was limited to the surgical group. Eighteen of 37 patients at risk showed evidence of spontaneous PSE, and this was considered severe in eight (22%). Five patients had moderate PSE, so that the combined incidence of moderate and severe PSE in this group was 35%.

It seems likely in all these studies that failure to demonstrate a difference in the <u>overall</u> incidence of encephalopathy is due to a higher incidence in the medically treated group of patients encephalopathy following gastrointestinal haemorrhage. Since recurrent bleeding is commoner in medically treated patients, this source of PSE counterbalances the incidence of <u>spontaneous</u> PSE in the shunted

group. It is only when severe spontaneous PSE is examined that differences due to the surgical procedure become apparent.

To summarize the above, it is reasonable to conclude that there is now good evidence from controlled studies that the incidence of PSE following total shunting is greater than that found in patients with comparable liver disease who do not have surgical shunts. Figure 6.1 (from Conn and Lieberthal, 1979) shows a schematic representation of the "epidemiology" of PSE in cirrhotic patients. The legend of this figure is self-explanatory, and highlights the rarity of chronic PSE in the absence of PCS. This conceptually useful scheme puts PSE into perspective in the context of cirrhosis.

1.2 The incidence of post-shunt PSE depends on the primary liver disease

We have already noted that in the 1950s reports were published of portacaval shunts in four patients with grossly normal livers (McDermott and Adams, 1954; McDermott et al, 1954; Hubbard, 1958) and that these patients within a few weeks or months developed episodic hepatic encephalopathy, fatty infiltration of the liver and hypoalbuminaemia. The conclusion of these studies was that the normal human liver was excessively sensitive to diversion of portal blood, and that patients with previously normal liver function would tolerate creation of an Eck fistula poorly. These reports have essentially not been supported by subsequent clinical experience. Indeed, for 20 years the risk of patients being considered for portal diversion has been assessed by the so-called Child classification, which is

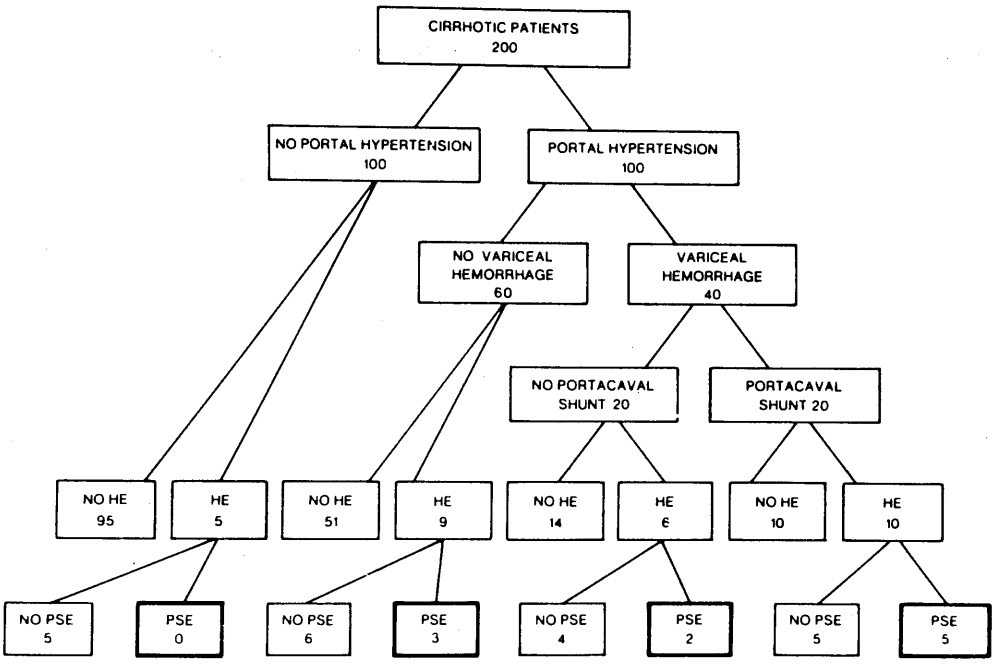


Figure 1-14. *Schematic diagram of the derivation of portal-systemic encephalopathy.* The scheme is based on 200 cirrhotic patients. None of the 100 patients without portal hypertension developed PSE, although a few may develop hepatic encephalopathy (HE). HE is defined as non-PSE and includes such non-nitrogenous causes as hypoglycemia, sedative- or tranquilizer-induced encephalopathy. Only a small fraction of those with portal hypertension who do not bleed from esophageal varices develop PSE. A large fraction of those who do bleed from varices and have portacaval shunts develop PSE. Thus, about 50% of PSE occurs in 10% of cirrhotic patients.

Figure 6.1

The "epidemiology" of portal-systemic encephalopathy.

(From Conn and Lieberthal, 1979)

based on the quality of hepatic function (Child and Turcotte, 1964). Numerous attempts to improve Child's prognostication have failed to make a marked impact (Campbell et al, 1973), although minor modifications suggested by Pugh (1973) have been adopted by some workers.

Starzl and his colleagues (1983) have attempted to explain what they call the "McDermott-Hubbard artefact" on the basis of loss of pancreatic hormones due to pancreatic resection carried out in all of these patients. While this suggestion would bring these observations into line with the experimental evidence for the importance of pancreatic hormones in the maintenance of liver mass and function, in relation to McDermott's and Hubbard's cases the proposal is highly speculative, since there is no available evidence suggesting diabetes or other pancreatic insufficiency in these patients. Further clinical evidence against this explanation has also been presented in a young patient treated by Linton who had a portacaval shunt constructed fo an acute portal vein thrombosis. Despite a normal liver and the absence of pancreatic resection, this patient developed PSE and protein intolerance (McDermott, 1983).

Contrary to these reports, there is clinical evidence supporting the safety of portal diversion in patients with previously normal livers. Much of this has been acquired in patients with glycogen storage disease or familial hypercholesterolaemia. Starzl has now followed nine patients with glycogen storage disease treated by shunting for 5-20 years (Starzl, Putnam et al, 1973; Starzl et al, 1983). Only one of these patients developed hepatic insufficiency and PSE eight

years after PCS. Similarly only one of 13 patients treated by PCS for familial hypercholesterolaemia has shown encephalopathy, and this was a single transient episode. Starzl comments on the absence of encephalopathy in 26 patients reported from other centres similarly treated. However, there is no doubt that these patients do have some impairment of hepatic function as a result of the shunt. Low grade elevations of serum transaminase and alkaline phosphatase are common, and blood ammonia levels when measured were always increased to and beyond the upper limits of normal (Starzl et al, 1983).

Another relevant group of patients is those with extrahepatic portal venous obstruction. Many of these patients develop a system of hepatopetal collaterals, driven by the portal hypertension which also produces the varices resulting in the need for surgical intervention. When some form of shunt is constructed the collateral flow to the liver is "stolen" through the shunt, and therefore results in some diminution of portal blood flow (Warren et al, 1980). Nevertheless, the majority of such patients are without encephalopathy during long-term follow-up (Fonkalsrud et al, 1974; Lambert et al, 1974). Similar results have been reported following shunting in children by Bismuth and his colleagues and by Alvarez and his colleagues (Bismuth et al, 1979, Alvarez et al, 1983).

Nevertheless, hepatic dysfunction and encephalopathy may occur following portal diversion in patients with extrahepatic portal block (Mikkelsen et al, 1965), and the PSE may indeed be reversed following disconnection of the shunt and restoration of hepatopetal flow (Warren et al, 1980). Voorhees and his

colleagues (1973) made the important observation that following PCS for extrahepatic portal block in children a high incidence of psychological and psychiatric abnormalities could be found, which might be a variant of PSE. While the significance of such changes in chronically hospitalized and investigated children is uncertain, the observation carries important implications.

A good prognosis after portal diversion has also been reported in patients with congenital hepatic fibrosis (Sokhi et al,1975)

Patients with schistosomiasis, a disease which produces a largely pre-sinusoidal portal block, ought to fare well following portal diversion, since hepatic function is often well maintained. However, this has not been found to be the case in follow-up of such patients (Goffi et al, 1968; Nel et al, 1974; Zeppa, 1974). The evidence from this quarter is complex, however, since the effects of schistosomiasis may not be purely mechanical, and other pathological factors may be involved in the deterioration of these patients' liver function.

In summary, while diversion of portal blood from the liver results in altered liver function, the incidence and severity of PSE in man following PCS is less in those patients with normal liver function before operation, and in whom it is well preserved after shunting. It remains unclear why deterioration in liver function following total shunting is such a variable phenomenon in man, and indeed why there is a marked species variation in the response to this procedure. The variability in man may relate to haemodynamic factors.

1.3. PSE may relate to the change in hepatic perfusion

Since Warren's observations in 1967 numerous attempts have been made to relate haemodynamic parameters, and in particular the degree of hepatopetal flow before shunting, to the outcome of portal diversion procedures. Much of the older work in this area was technically unreliable, particularly studies based upon angiography (Malt, 1976). Indeed, numerous attempts to find haemodynamic parameters which correlate with the outcome of portal diversion surgery have been largely fruitless (McDermott, 1972; Reynolds, 1974a and b). Attempts to maintain hepatopetal portal flow by side-to-side portacaval shunt (of which the central spleno-renal shunt is a variety) have been shown to be futile, and performance of this type of shunt has no clinical advantage over end-to-side PCS (Bismuth et al, 1974; Reichle and Owen, 1979; Adson et al, 1984). The origal report by Drapanas (1972) suggested that after a meso-caval interposition ("H") graft the PSE rate was as low as 11%. This has never been substantiated by other workers. Mulcare (1984) in New York in a retrospective survey reported a 32% incidence of PSE "of sufficient gravity to require active medical therapy": this did not appear to relate to pre-operative liver function. Resnick et al (1984) of Langer's group in Toronto reviewed 30 survivors of 47 mesocaval shunts: 10 (33%) had mild PSE and 6 (20%) severe, defined as "disabling or requiring hospitalization". There have been two controlled trials of mesocaval shunt against conventional PCS. Malt et al (1978) found no advantage for mesocaval shunt in emergency cases, though the mortality rate was high (73%). Stipa et al (1981) in electively shunted Child's A

and B patients also showed little difference in survival or in overall PSE (43-44%), though the rate of severe PSE was unusually low in his series (1/46 patients).

The original observation by Drapanas (1972) that mesocaval shunt maintained hepatopetal portal flow has also not been confirmed by other workers, and these results carry an important lesson on the dangers of haemodynamic interpretation of angiographic data. Coeliac or superior mesenteric injections may show filling of the portal vein, but not distinguish between hepatopetal and hepatofugal flow - a misinterpretation termed "portal pseudoperfusion" by Warren's group (Fulenwider et al, 1979). Splenic arterial injection may clarify the situation and demonstrate total portal shunting (Reznick et al, 1984).

Of course, it must also be noted that in some patients with liver disease and portal hypertension portal venous inflow makes a very small contribution to hepatic perfusion even before surgical portal diversion (Lieberman et al, 1978)

While the majority of authors have attempted to study alterations in portal flow following total or partial shunts, Burchell and his colleagues made important observations regarding changes in arterial flow after shunting (Burchell et al, 1974). These workers demonstrated that following total PCS there was a variable increment in the volume of hepatic arterial flow to the liver. Follow-up studies demonstrated that those patients who had an increment of hepatic arterial flow greater than 100 ml/min suffered a very low incidence of encephalopathy, while those with a smaller increment in arterial flow had a significantly higher

incidence (Burchell et al, 1976). This is important evidence relating the volume rather than the character of hepatic blood flow to the incidence of encephalopathy. Maillard and his colleagues (1974) reported the use of arterialization of the portal venous stump in association with PCS in order to avoid the complication of PSE. While this was to some extent effective, it carried a high rate of complications including intrahepatic cholestasis and marked portal venous sclerosis, and does not appear to be of clinical value. Nevertheless, this work emphasizes again the importance of maintaining total hepatic blood flow in the presence of portal-systemic diversion.

Thus there is clinical as well as experimental evidence relating maintenance of total hepatic blood flow to a lowered incidence of PSE. It is this concept which led Warren and his colleagues to the development of the selective shunt, which would decompress the oesophagogastric variceal area while leaving intact the residual portal flow to the liver. Finally in this Section we will review the clinical evidence for the efficacy of these shunts.

1.4 Selective shunting produces less PSE

The distal spleno-renal (Warren) shunt is illustrated in Figure 6.2. The concept of this operation is that blood from the gastro-oesophageal varices is shunted via the splenic hilus into the vena cava by a spleno-renal venous anastomosis. The coronary veins and other collaterals are divided, so that portal venous flow into the portal tracts of the liver is maintained from a "right-sided" portal compartment which remains under

(pathologically) high pressure. Separation of this high pressure portal compartment from the oesophagogastric region renders it harmless with respect to producing gastrointestinal bleeding. Warren et al (1974) showed that this did indeed lead to better preservation of hepatic function as measured by maximal rate of urea synthesis, although the most recent report from Warren's group shows deterioration in galactose elimination capacity during follow-up of patients with distal spleno-renal shunts (Warren et al, 1986). Nevertheless there is now evidence from controlled clinical trials of the distal spleno-renal or Warren shunt that the incidence of PSE is lowered (Rikkers et al, 1978; Reichle et al, 1979; Resnick et al, 1979; Langer et al, 1980). Langer's results showed that only 3/22 patients surviving a selective shunt procedure had significant encephalopathy, classified as mild in all cases. In contrast, 14/28 patients with end-to-side PCS had encephalopathy, 9 to a mild degree and 5 (18%) severe. These results were statistically significantly different. Conn and Lieberthal (1979) combined the available data from Warren's, Langer's, and the Boston-New Haven Liver Group (Resnick and Conn) and showed an identical survival between selective and portacaval shunts in these trials, but a significantly lower incidence of PSE of all grades (7/50 vs 20/52, $p < 0.025$), and particularly of severe degree (1/50 vs 12/52, $p < 0.005$). Reichle et al (1979) also showed a significantly lower incidence of PSE after Warren shunt compared to mesocaval shunt. Adson et al (1984) reviewed the Mayo Clinic's experience of 71 Warren shunts over 10 years. The operative mortality rate was 4%, with a 76% actual 3 year survival and a "probable" shunt occlusion rate of 10%. The

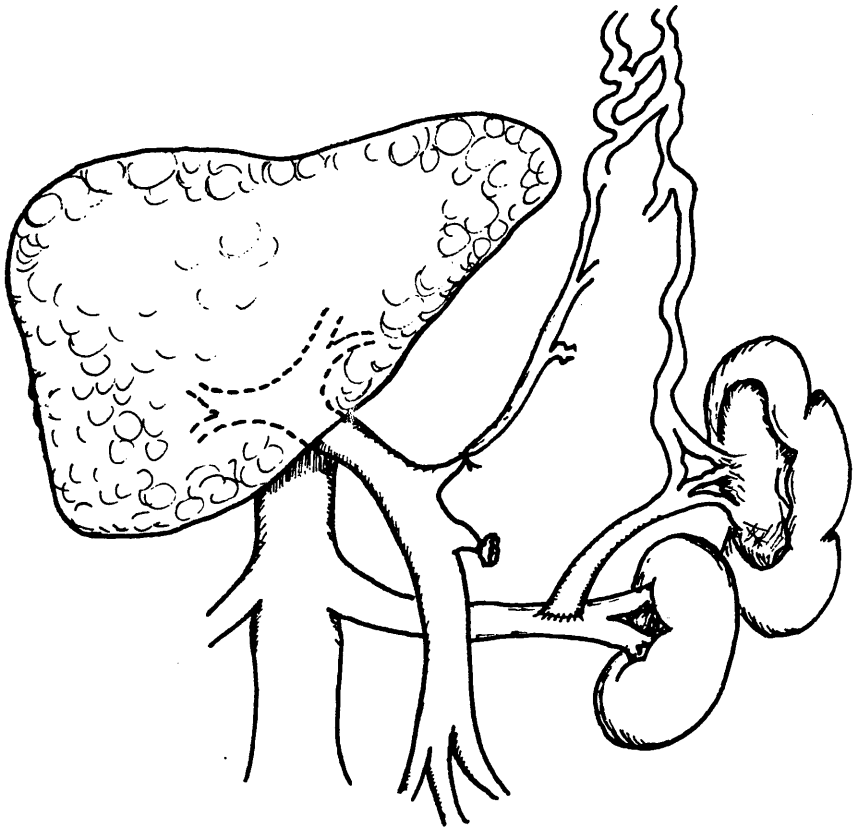


Figure 6.2

The distal splenorenal (Warren) shunt.

incidence of PSE was "5.6% or 7%", the latter figure including one patient who "becomes confused when drunk"!.

In contrast, Marni et al (1981) showed no advantage of selective over total shunting in terms of encephalopathy. Mikkelson reported the interim results of an on-going controlled trial of Warren shunt versus PCS in Los Angeles (discussion of Adson et al,1984). In a trial restricted to Child's A and "high B+" patients, he reported no overall difference in PSE between the groups. In both of these studies, selection of the lowest risk patients for inclusion may have considerably influenced the outcome.

Thus maintenance of hepatic blood flow, in this instance by continued portal perfusion, protects against deteriorating liver function and the development of PSE. However, in the case of the Warren shunt, the mechanism may not be quite so clear-cut. The presence of one splanchnic venous bed perfusing a diseased liver under high pressure within the abdominal cavity alongside a second low pressure splanchnic venous bed draining a large part of the foregut into the left renal vein seems unlikely to be an arrangement which could be maintained in the long term. Indeed Maillard and his colleagues (1979) have shown that there is gradual loss of selectivity of the shunt. These workers have shown that the distal spleno-renal shunt carries a high flow which is largely driven by the splenic arterial input. This may protect the spleno-renal anastomosis from thrombosis, but also produces a "steal" effect of the shunt because of its arteriovenous fistula-like character. However, this spontaneous conversion from a selective to a total shunt may be very

gradual: Warren (1983) showed angiographically that 9/11 patients in a randomized study who agreed to have follow-up angiography had continued hepatic perfusion on superior mesenteric injection at a mean of 7.4 years post-shunt. It may be that the gradual loss of hepatic perfusion allows time for compensatory adjustment to take place in the liver, either metabolic or haemodynamic, with increase of hepatic arterial flow. In fact, Reichle and Owen (1979) showed that, while portal venous perfusion was maintained in the short term following Warren shunt, hepatic arterial flow actually decreased slightly: following mesocaval shunt, both portal and arterial flow decreased.

There is another factor in selective shunts which may contribute to the low incidence of PSE. It has been shown that following a total portal shunt the reduced portal capillary pressure in the intestines results in increased absorption of ammonia and other metabolites (Price et al, 1966; Harrison et al, 1977). This phenomenon had been observed by Warren at the time of his original description of the selective shunt (Warren et al, 1967), and re-emphasized recently (Warren et al, 1982). Thus maintenance of a high splanchnic venous pressure during the long adaptive period may lead to important reduction in toxic absorption from the gut, in addition to the protective influence of well maintained hepatopetal portal flow.

Despite all of the above discussion, the precise reasons for the difference in PSE between selective and total shunts remain unclear. Nonetheless, the improved clinical results from selective shunting have led many workers to adopt the procedure,

and incomplete understanding of the mechanisms underlying its efficacy have not been a bar to its clinical use.

However, not all surgeons have accepted the Warren shunt without reservation. It is a technically more exacting procedure, possibly less suitable for emergency use, and may be attended by a lower long-term patency rate than conventional PCS. Bismuth and his colleagues have continued to prefer end-to-side PCS, and in the most favourable groups (Child's A, non-alcoholic cirrhotics) has achieved an 82% 5-year survival, all deaths being due to unrelated causes, with no significant severe chronic spontaneous PSE. For unselected patients the survival was 69%, with severe chronic PSE in only 5% (H Bismuth, personal communication).

2. WHY DOES PCT DIFFER FROM PCS? - THE HEPATOTROPHIC CONTROVERSY

Some of the history of this controversy has already been reviewed. In this Section the points which are of key importance and their relevance to the PCT model will be highlighted.

The rapidity with which atrophy follows Eck fistula in animals, being 90% complete within three to four days, has only been recently appreciated (Starzl, Porter and Putnam, 1975). The early time course of this process has not been studied in detail in rats, but in the present experiment there was already established liver atrophy at 72 hours after PCS, maintenance of relative liver mass in PCT animals. The observation of this atrophy by Rous and Larimore (1920) led to the first "modern" hepatotrophic hypothesis: these authors suggested that "the liver has no essential activity.... none on which its maintainence depends.... that is not intimately connected with substances derived from organs drained by the portal system". In 1940 Mann concluded that the volume of blood presented to the liver was the most important factor, stating that "restoration of the liver depends upon..... the quantity of the portal blood". These early studies were based upon the response of the Eck fistula dog to partial hepatectomy. The flow hypothesis was generally accepted as being confirmed by the work of Child and his colleagues (1953) in which portacaval transposition allowed normal restoration of liver mass following hepatic resection in the dog, and also circumvented most of the adverse effects of Eck fistula. Further elucidation of the controversy awaited the

development of studies in experimental liver transplantation during the 1960s. Following observations that an auxiliary heterotopic liver transplant underwent rapid atrophy, Marchioro and his colleagues (1965) showed in a key paper that atrophy of the transplant could be avoided and inflicted instead upon the host liver by diverting splanchnic venous blood through the graft.

These experiments, and refinements which were produced by other workers, suffer from the serious flaw of uncertainty regarding the relative total perfusion rates of the two livers. Moreover, in homograft experiments immunological rejection of the transplanted liver might have led to unfair "competition". This problem led to the development by Marchioro and his colleagues in Starzl's laboratory of the first of several "split transposition" models (Marchioro et al, 1967). In these experiments splanchnic venous blood was allowed to perfuse the portal tracts of one lobe of the liver, while inflow to the opposite lobe was replaced with blood from the vena cava, as in the standard "complete" PCT. All of these experiments led to hyperplasiain the lobe supplied with splanchnic blood and atrophy of that supplied with systemic blood. The ultimate refinement of this model was produced in Starzl's laboratory in 1973 (Starzl, Francavilla et al, 1973). In this split transposition model one lobe of the liver was provided with blood draining the pancreas, stomach, duodenum and spleen, while the other was perfused with blood from the superior mesenteric vein. In this case the lobe provided with blood from the pancreas and other upper abdominal organs remained healthy, while the other lobe atrophied. Specifically, hepatocyte size

was measured and shown to be different between the two lobes, and there were also major biochemical differences in glycogen and lipid content, and in activity of glucokinase, cyclic AMP and active phosphorylase. Further refinements of these experiments suggested the primacy of insulin in liver maintenance, and in 1975 and 1976 Starzl showed that insulin (but not glucagon) infusion into one lobe of the liver of a dog subjected to end-to-side PCS would protect that lobe from atrophy and encourage hepatocyte proliferation.

This work from Starzl's laboratory has been pivotal in our thinking about hepatotrophic factors. There are, however, some possible problems with this work, and not all these findings have been confirmed. The possibility of different rates of flow in the split transposition model was a major one, particularly since the model requires a long venous graft to one branch of the portal vein. In order to resolve this problem, Mathie et al (1979) in our own laboratory reproduced Starzl's split transposition model, and measured blood flow in the two lobes of the liver: hepatic tissue perfusion in both lobes of the liver was equal, confirming the validity of Starzl's observations.

Recently Castaing et al (1982) in Bismuth's laboratory in Paris studied various types of portal diversion in the rat. These included PCS, PCS with arterialization, PCT, mesocaval anastomosis (in which only intestinal blood was diverted to the systemic circulation) and pancreatico-splenocaval anastomosis (in which intestinal blood still flows through the liver while pancreatic and splenic blood are shunted). Among these different types of portal venous diversion only PCS produced significant

liver atrophy by 14 days after operation. Specifically, suppression of the pancreatic blood supply to the liver by pancreatico-splenocaval anastomosis produced no more liver atrophy then did diversion of mesenteric blood, PCT or arterialization. These workers also cite similar results observed by Hess, ten weeks after total or selective diversion. There are two major differences in the approach of Bismuth's and Starzl's group. Bismuth measured the effect of portal diversion on the size of the whole relative liver mass at 14 days after operation, while Starzl's observations were made on the size of the liver cells at four days. Secondly, in Starzl's split transposition models, a degree of functional competition is created between the two lobes of the liver, a situation analogous to that previously observed in relation to the growth of heterotopic auxiliary liver grafts (see above). Such functional competition might strengthen the minor advantage conferred on one lobe by the perfusion of insulin and thus overemphasize the hepatotrophic role of this hormone.

Another important aspect of Castaing's paper lies in the relationship demonstrated between liver blood flow and liver mass. Only PCS produced a major decrease in total estimated hepatic blood flow (a reduction from 4.4 to 2.6 ml/min/100 g body weight), and this was also the only group with major liver atrophy (relative liver weight reduced from 3.18% to 2.18%). Rats subjected to PCS or liver arterialization had a normal estimated hepatic blood flow and no major liver atrophy. Thus hepatic blood flow may still retain a role in maintenance of liver mass. This work from Bismuth's laboratory also showed that

post-shunt encephalopathy (assessed by measurement of the level of wakefulness) only occurred in association with total diversion of portal blood and liver atrophy. This is an observation of considerable importance which will be discussed further in Section 3 below.

It is apparent that there are numerous problems inherent in interpretation of data obtained from whole animal models in this difficult area. This has led several groups to use cell or tissue culture preparations in order to obviate some of these problems. There is in fact good evidence for a major trophic role of insulin obtained from such studies. Ozawa's group in particular have examined the role of hormones on mitochondrial oxidative metabolism both in the whole animal and in culture, and have confirmed an important role for insulin (Ozawa et al, 1971; 1973; 1974). Detailed consideration of these techniques is beyond the scope of this Thesis, although further reference will be made to the impact of cell culture techniques in liver regeneration at the end of this Section.

In view of much of the foregoing evidence one might not expect that liver mass and function should be preserved at a completely normal level after PCT. Several authors have demonstrated subtle abnormalities following PCT. Pector et al (1975) showed alterations in the hepatic mixed function oxidase system following both PCS and PCT. (It is interesting to note that after PCS cytochrome P-450 activity in the liver can be maintained at normal levels by administration of clofibrate, a potent enzyme inducer (Cortesse et al, 1980). This manoeuvre also maintained liver mass, and prevented the changes of

encephalopathy). Nevertheless, the present studies and those of others working in dogs (Child et al, 1953; Heer et al, 1960; 1963; Summers et al, 1956; Turney et al, 1966; Furtado, 1963) and rats (LeCompte et al, 1973; Engelbrecht et al, 1981; Castaing et al, 1982), confirm that liver mass and morphology and a number of crucial metabolic functions of the liver are maintained close to normal after PCT. In any event the contrast between PCT and PCS is sufficiently great to allow a valuable baseline from which to make comparisons of other effects, and specifically in this work the effect on PSE.

Finally, it remains necessary to reconcile the flow and hepatotrophic theories in relation to PCT or indeed to portal arterialization. If trophic substances (such as insulin) are not substantially metabolized in peripheral tissues, and if there is no competition for these substances from other native organs or from a second engrafted liver, then these moieties will reach the liver by way of systemic recirculation through the portal tract. In this event although the concentration reaching the liver will be less than that seen in the intact animal, the rate of delivery will be proportional to the portal tract inflow. In the final analysis, this may well account for all of the differences seen in various experimental models, and may indeed be sufficient to account for the minor changes after PCT observed in the present studies.

It is important to distinguish factors which regulate liver mass from those which initiate or control the process of liver regeneration. Failure to appreciate the distinction between these two phenomena led to much of the confusion generated by the

experimental studies of the 1940-1960 era. The search for a factor which might initiate or control the process of regenerative hyperplasia in the liver following partial hepatectomy spans at least two decades. An intrinsic inhibitory factor controlling liver growth was suggested by the finding that regenerative activity after partial hepatectomy was reduced by intraportal infusion of fresh serum from an intact animal (Bradbrook et al, 1973). However, other studies suggest that regenerating liver itself produces a circulating factor which has a stimulatory effect upon cell replication in the hepatocytes of another target animal. The work carried out by Terblanche in Starzl's laboratory (Starzl et al, 1979; Terblanche et al, 1980), and subsequently extended in his own laboratory (van Hoorn-Hickman et al, 1981; Kahn et al, 1982), showed that a stimulatory substance could be extracted from the blood of a partially hepatectomized animal which was able to enhance regenerative activity in test animals subjected to PCS or 44% partial hepatectomy. In addition, plasma taken from the perfusate of an isolated partially hepatectomized liver could also enhance regeneration in a test animal with a PCS. Moreover, the regenerative activity in an intact or partially hepatectomized auxiliary liver graft was also found to be "transmitted" to the portacavally shunted host liver.

The most exciting development in this area in recent years has been the identification of an "hepatic stimulator substance" (HSS), isolated and partially characterized by LaBrecque and Pesch (1975). The substance appears to be a protein of molecular weight 10,000-15,000, and its effects are organ specific

(including cultured hepatocytes and hepatoma cell lines), but not species specific. It would have been scarcely possible to conceive at the time that the present studies were commenced that we should already be so close to witnessing isolation of this fundamental Promethean molecule. Its identification clears the way for our comprehension of the difference between initiating or controlling factors and those which are permissive in the sense that they create a favourable metabolic environment for liver cell growth. Almost certainly hepatic blood flow and the trophic substances extensively investigated by Starzl fall into latter this category of permissive factors. Certainly the work of LaBrecque has shown that the presence of insulin is neither obligatory for nor synergistic with the effects of HSS in cell culture systems (LaBrecque, 1979). The apparent silence on this vital work from LaBrecque's laboratory in recent years is due to the search for a sensitive and specific hepatocyte tissue culture system which can act as a reliable biological assay for HSS (LaBrecque, personal communication): further developments will be awaited with great interest.

Our own work in relation to PCT and the regenerative response (Guest et al, 1977) confirms the separation between permissive hepatotrophic factors and the underlying fundamental mechanism of liver regeneration. In these experiments, both the response of hepatocytes with an early wave of mitosis at 21 hours after partial hepatectomy and the response of the whole liver in terms of restoration of liver mass proceeded at the same rate in PCT rats as in control animals. PCT seems to "reset" the "normal" liver mass as a function of body mass. In these

experiments the restored liver mass following two thirds partial hepatectomy at 3 weeks after PCT was by 6 weeks the same as that expected following the normal process of slowly progressive liver atrophy in PCT animals without liver resection. Other work from our laboratory has shown that this normal regenerative response seen in PCT rats is also independent of changes in hepatic tissue perfusion seen immediately after partial hepatectomy in normal rats (Rice et al, 1977).

In summary, there has been increasing recognition that regenerative hyperplasia and maintenance of liver mass are related but not identical phenomena, and that the roles of blood flow and of portal trophic substances may enjoy a similar degree of independence. Thus in the space of two decades controversy has turned to reconciliation in the flow versus hepatotrophism debate.

3. THE RELEVANCE OF PORTAL DIVERSION MODELS IN PSE

The perfect model for chronic portal-systemic encephalopathy has not yet been produced. Models of acute liver failure or of chronic liver disease induced by surgery or by drugs correspond only poorly to the patient with PSE secondary to cirrhosis and portal hypertension. However, in this section the evidence connecting PCS with the PSE syndrome in animals will be briefly reviewed, and the results of the few studies which have examined PCT or similar models will be outlined.

The historic work of Eck and of Hahn (Chapter II) established the capacity of PCS in the dog to produce hepatic coma, and the relation of this encephalopathy to dietary protein has been confirmed in more recent times (Condon, 1971). Starzl et al (1983) have reviewed the species factor in encephalopathy, and note that subhuman primates may develop a fulminant Eck fistula syndrome, with fully developed histological changes in the CNS. The response of pigs has varied from severe hepatic failure and encephalopathy within a few days in the hands of some workers (Cuschieri et al, 1974; Hickman et al, 1974), to survival for several months in others (Chase and Morris, 1976). However, it has already been noted that in the rat spontaneous coma rarely occurs following PCS. It is unknown why there should be this wide species variation in response to PCS, but differences in hepatic metabolic reserve following portal vein ligation have also been observed between species (Ozawa et al 1973).

Thus it is to more sophisticated evidence that we must turn

to assess the value of portal diversion models in the rat. It is possible to induce coma in PCS rats by administration of ammonium salts by mouth (Hindfeld et al, 1977), or by feeding with ammonium cation exchange resins (Pilbeam et al, 1983). These findings are in keeping with the known ammonia intolerance of shunted animals, and indeed the ammonia tolerance test has been used in man as a valuable indicator of the patency of a shunt (Conn and Lieberthal, 1979). Ammonia tolerance was shown to be better following PCT than following PCS in dogs (Brown et al, 1967).

PCS animals do show changes in spontaneous EEG recording (Herz et al, 1972), but similar studies have not been reported for PCT. More sensitive than the spontaneous EEG is the recording of visual evoked potentials in response to flash of light . The use of this technique in rabbits (Schafer and Jones, 1982) and in rats (Zeneroli et al, 1982) has allowed differentiation of abnormal electrical patterns in ammonia or tryptophan induced coma and encephalopathy due to galactosamine-induced fulminant hepatic failure. EEG patterns and other less specific methods of examining nervous activity do not allow differentiation of these subtleties, and this is a valuable technique which has yet to be applied to PCS and PCT in the rat.

Altered sleep patterns have been seen in PCS rats, with loss of the normal day-night rhythm of activity, maximum by 1 month after operation (Campbell et al, 1979). The technique adopted by Bismuth's group in Paris has been to examine the level of wakefulness of animals by measuring the reactivity of the

reticulocortical and reticulothalamocortical systems to electrical stimulation of the brainstem reticular formation during slow wave sleep. The minimum voltage level which must be applied to the brain stem reticular formation by means of implanted electrodes to produce cortical and behavioural awakening was measured in various groups of experimental rats (Castaing et al, 1982; Cortesse et al, 1980). The level of wakefulness has been found to be consistently increased (that is to say the magnitude of the stimulus required to elicit awakening was reduced) two weeks after PCS. This group studied the same phenomenon in rats subjected to PCT by the technique of Lecompte et al (1973). They found that PCT rats showed no change in cortical awakening, and that similarly animals with portal arterialization were normal in this respect (Castaing et al, 1982). These workers also measured hepatic blood flow and liver size in these and other models, and concluded that for the production of encephalopathy both portal-systemic shunting and liver atrophy were required. The same workers had demonstrated that administration of clofibrate to PCS rats prevented the expected liver atrophy and also prevented encephalopathy (Cortesse et al, 1980). These important results emphasize the role of what these workers term "functional hepatic mass" in the mechanism of encephalopathy following portal diversion in the rat, and lend considerable weight to the validity of such models.

Other forms of functional neurological assessment such as open field behaviour (Tricklebank et al, 1978) and the use of light activity boxes has also been described for PCS animals

(Rigotti, Jonung et al, 1985), but not for PCT. This type of testing is, at least conceptually, superior to models involving the production of acute coma states in attemting to parallel chronic PSE associated with portal diversion in man. Unfortunately, the complex methodology of these procedures has resulted in a scanty literature on the subject.

The histological changes in the CNS following portal diversion have been amongst the most consistent of observations in all species. The background of these changes was reviewed in Chapter IV, and the specific role of the glial cells in PSE will be further discussed below (Section 5). Here it is sufficient to note that our own studies have confirmed the changes in the astrocytes of the dentate nucleus and in the Bergmann cells reported by other workers after PCS. We have shown an important reduction in the incidence of these abnormalities following PCT, an observation which had not previously been made. Since the completion of our work Vassanelli and his colleagues in an abstract presented to the European Surgical Research Society demonstrated that PCS with portal revascularization either by our technique of PCT or by anastomosis with the right renal vein "produced a sharp improvement (that is in the histopathologic findings in the rat brain), as the lesions found were very slight". They also stated that "this improvement could not be differentiated quantitatively or qualitatively from that observed with hepatic arterialisation". This abstract is valuable confirmatory evidence of the protective effect of PCT seen in our studies, although these authors did not assess astrocyte changes.

The numerous biochemical changes associated with PCS have been partly discussed elsewhere. To cite alterations in CNS chemistry as evidence of the validity of the PCS/PCT model would be to beg the question of the value of portal diversion in the investigation of PSE. In the review of CNS chemistry which will follow, however, it should become clear that custom and usage have more than honoured the PCS rat model in the past, and there appears to be little reduction in the flow of papers on this subject in recent years.

4. PUTATIVE FACTORS IN THE PATHOGENESIS OF PSE

The liver occupies a central role in the maintenance of metabolic stability, with widespread effects on carbohydrate, protein, and hormone metabolism, and this diversity of hepatocyte functions is reflected in the protean metabolic effects of liver disease. We have already seen that diversion of blood flow from the liver must be regarded as a specific form of insult, frequently iatrogenic, which will produce a wide range of hepatic and systemic results. A number of possible agents associated with PSE were discussed in Chapter V, and is appropriate in this final discussion to examine the role of liver function in relation to some of these putative agents.

4.1 Ammonia

This was the first of these agents to be postulated, and there is extensive evidence to demonstrate the importance of the liver in ammonia metabolism. The most important pathway for ammonia utilisation is urea synthesis in the liver by incorporation into carbamyl phosphate which subsequently enters the urea cycle (see Figure 5.1): the liver is the only organ which synthesizes carbamyl phosphate and urea. Reduced urea cycle enzymes in patients with cirrhosis and portal-systemic shunting have been demonstrated by Khatra et al (1974). There are a number of inherited hyperammonaemic syndromes due to deficiencies of the urea cycle enzymes, including the key enzyme carbamyl phosphate synthetase, and those which are not lethal in infancy result in profound neurological disturbances, including

postural abnormalities and coma with a variety of less severe neurological and neuropsychiatric symptoms in the lesser forms of the disease (Shih, 1976; Flannery et al, 1982). In these patients typical Alzheimer Type II cells have been described in the central nervous system (reviewed by Conn and Lieberthal, 1979). Similar defects have been investigated in the dog (Strombeck et al, 1975). We have noted that following PCS ammonia tolerance is impaired (Kirsh et al, 1964) and Turney et al (1966) showed that tolerance was better following PCT than PCS in dogs. These workers postulated that the function of the hepatic parenchyma was a more important determinant of blood ammonia levels in the dog than portal-systemic shunting. Our present study showed blood ammonia levels which were equally elevated following PCS and PCT, and this result is in keeping with the work of Castaing et al (1982). Moreover, these workers showed no evidence of encephalopathy in rats with PCT or liver arterialization despite marked elevation of blood ammonia levels. Thus while ammonia almost certainly plays a central role in some of the metabolic processes associated with PSE, our studies and the confirmatory ones from the French group do not lend support to the primacy of ammonia, but rather emphasize the importance of other factors which relate to functional hepatocellular mass. Ammonia remains a valuable index of the degree of portal-systemic shunting: Orloff and his colleagues (1963) demonstrated that ammonia intolerance was dependent upon the shunt size, and shunts of greater than 2 cm in diameter in dogs led to a maximal increase in the peak ammonia and duration of elevation during an ammonia tolerance test.

It is particularly interesting in the present studies that tissue ammonia levels were disproportionately elevated in some areas of the brain following PCS, despite identical plasma levels in PCS and PCT. This result may highlight the important selective regional nature of both biochemical and histological events in the CNS in PSE, and also raise the possibility that either there is a regional change of blood-brain barrier permeability to ammonia or local alterations in ammonia metabolism following PCS. Some of the collateral evidence concerning this will be reviewed below.

4.2 Amino acids

Amino acid changes following PCS and PCT have formed an important part of this work. Our results suggest that with respect to PSE the most important difference between the PCS and PCT animals lies in the elevation of aromatic amino acids (AAAs) in the former group. It was possible to abolish differences in the branched chain amino acids (BCAAs) by pair-feeding without influencing the difference in AAA levels or the difference in the incidence of CNS changes. The elevated levels of AAAs are not surprising, since there is little extrahepatic metabolism of these molecules. Rigotti and his colleagues in Padua (1982) examined the effect of hepatic arterialization along with PCS. They also found that the plasma levels of TYR and PHE were elevated after PCS but normal after PCS with arterialization, four weeks after operation. Both groups of animals in this study showed reduced levels of BCAAs.

The reduction in BCAAs following PCS requires more

explanation than the elevation of the aromatic group. According to the Munro hypothesis (Munro et al, 1975), this reduction is associated with the hyperinsulinism seen in cirrhotic patients and in animals after PCS. These workers originally proposed that hyperinsulinism resulted in increased uptake and metabolism of BCAAs for gluconeogenesis in skeletal muscle, and there is experimental evidence demonstrating an inverse relationship between insulin levels and plasma BCAAs (Soeters et al, 1977). However, incorporation into muscle could not adequately explain the isolated decrease in BCAAs, and Soeters went on to demonstrate that incorporation of LEU into fatty acids in adipose tissue from PCS rats was greater than that in normal animals (Soeters, 1979). Insulin stimulates the uptake of LEU into adipose tissue (Goodman, 1964). Moreover, in rats with streptozotocin-induced diabetes BCAAs are greatly increased in both normal and PCS animals (Ziparo et al, 1979).

However, plasma insulin and glucagon are clearly not the only important factors in the reduced BCAAs. In patients with normal liver function and spontaneous portal-systemic shunts Iwasaki et al (1980) reported normal insulin levels, though BCAAs were reduced. Moreover, it has been shown that in cirrhotic patients the effect of insulin on perpheral uptake of BCAAs is reduced (Marchesini et al, 1983). One further possible factor is the high plasma concentration of ammonia, whose detoxification consumes alpha-ketoglutarate which is supplied by the BCAAs: this is supported by the findings of Marchesini et al (1983) that plasma BCAAs fell after ammonia tolerance tests in cirrhotic patients. These findings have not been confirmed, and one group

have reported contrary results (Jonung, 1985)

It is not possible from the results of the present studies to implicate hyperinsulinaemia as the principal agent in the reduction of BCAAs, since elevated insulin levels were seen in ad lib fed PCT rats who had normal BCAA levels. The present pair-feeding experiments are strongly suggestive of a major dietary factor, since BCAA levels were reduced in both PCT and control rats when pair-fed to the "anorexic" levels of the PCS animals. Skeletal muscle has been shown to degrade more BCAAs in starvation, associated with low insulin levels (Odessey and Goldberg, 1972). Unfortunately, no plasma hormone levels were available from the present pair-feeding studies to evaluate this relationship.

An important difference between our results and those reported by Fischer and his colleague lies in the relative increases in insulin and glucagon, which were in the present studies equimolar. The mechanism for elevated levels of these hormones in liver disease with portal-systemic shunting may be increased secretion or diminished degradation. Studies of the insulin precursor c-peptide have suggested that diminished degradation, due to hepatocellular insufficiency or to shunting of portal blood, is a major factor (Johnston et al, 1977). The weight of clinical evidence seems to favour the importance of hepatocellular dysfunction: Smith-Laing et al (1979) showed that in patients with portal vein thrombosis there was abnormal glucose tolerance but a relatively well maintained insulin response, with evidence of only minor impairment of insulin degradation at the highest secretion rates. This result was in

distinction to patients with cirrhosis, who had markedly impaired insulin degradation. However, there is also evidence in support of the contrary view. High plasma glucagon levels in patients with chronic liver failure correlate with the degree of portal-systemic shunting, but not with liver function (Sherwin et al, 1978; Dudley et al, 1979). Glucagon levels are much more variable in cirrhosis than those of insulin (Johnston et al, 1982). There is also evidence of hypersecretion of glucagon in shunted animals (van Hoorn-Hickman et al, 1979, 1980; Soeters et al, 1977).

Hormone levels were available from only a small number of animals in the present experiments, but the evidence of equimolar elevation of insulin and glucagon in both PCS and PCT groups would favour the primacy of shunting rather than hepatocellular dysfunction. Of course, species differences may be important, and most of the evidence in relation to hormone imbalance has been obtained in dogs or in man. Nevertheless, from our own experiments we are forced to conclude that differences in amino acid profile may arise between PCS and PCT rats despite an identical hormonal environment, so that we cannot postulate a hypercatabolic state as a result of disproportionate hyperglucagonaemia as the prime cause of the deranged amino acid profile seen after PCS. Fischer's group have suggested that a new steady state of amino acid balance emerges in patients with liver failure, with increased concentrations of those amino acids which rely for their homeostasis on hepatic metabolism under the control of glucagon. Elevated glucagon levels drive the liver to maintain metabolism of the AAAs at maximum capacity. When liver

function deteriorates further and the AAA levels increase, further failure of their catabolism by the liver may reduce the glucose output, with a consequent reduction in insulin production resulting in the elevated glucagon to insulin ratio seen when chronic stable liver failure progresses to acute encephalopathy. These postulated changes form the background of the "catabolic state" which has been proposed in severe liver failure with coma.

4.3 Amino acids and the CNS

Central to the amino acid/false neurotransmitter hypothesis is the concept that altered amino acid metabolism in chronic liver failure induces changes in the rate of entry of amino acids and other substances across the blood-brain barrier into the CNS where they exercise metabolic effects resulting in neurotransmitter abnormalities. The literature on the subject of blood-brain transfer of these moieties has become immense and complex in the last five to ten years, and it is therefore necessary to be selective in discussion. Since there are important differences between chronic PSE and acute liver failure, I have tried to avoid discussion of results relating to acute hepatic coma models, except where these results are of clear relevance to the topic. It is important to make this distinction because while much of the hypothesis implicating altered plasma amino acid patterns in the development of PSE assumes relatively physiological conditions for the operation of the blood-brain barrier transport mechanisms, it may be that models of acute hepatic failure produce gross changes resulting in different permeability and uptake patterns. These changes

TABLE 6.1

Putative neurotransmitters in the central nervous system

COMPOUND	SYNTHESIS	INACTIVATION
Acetylcholine	Acetylation of choline	Acetylcholineterase
Noradrenaline	β-hydroxylation of dopamine	1. Re-uptake 2. Monoamine oxidase 3. Catechol-o-methyl transferase
Dopamine	Hydroxylation of TYR	1. Re-uptake 2. Monoamine oxidase 3. Catechol-o-methyl transferase
Serotonin	Hydroxylation of TRP	1. Re-uptake 2. Monoamine oxidase
Histamine	Decarboxylation of histamine	Methylation ?Monoamine oxidase
Excitatory amino acids (GLU, ASP)	——	Uptake by glial cells Amination of GLU -> GLN (see text)
Inhibitory amino acids (GABA)	Decarboxylation of GLU	Uptake Transamination
Substance P (?)	——	?Proteases
Prostaglandins (?)	Fatty acids	——

will be considered below in discussion of the blood-brain barrier.

Following the original papers defining the amino acid/false neurotransmitter hypothesis (Fischer and Baldessarini, 1971; Fischer and James, 1972) Fischer's group and others have produced a considerable body of work elucidating the transport mechanisms of amino acids into the CNS. James et al (1976) showed that following PCS in the rat plasma and brain TYR were both elevated, but the increase in brain levels was disproportionate to that in the plasma. There was a significant correlation between plasma or brain TYR levels and the relative liver weight of the animals, confirming the importance of "functional liver mass". These studies also showed that brain octopamine levels increased as the relative liver weight decreased, and there was a positive correlation between brain octopamine and brain TYR. This last observation would be expected because of an increase in tyramine synthesis with the increasing TYR levels (see Figure 5.6). It was presumed at this time that the disproportionately high rate of uptake of TYR into the brain from the plasma was due to low plasma levels of BCAAs seen in Fischer's previous studies. This was an assumption of all the studies up to 1978 to 1979, the time at which the work in this Thesis was concluded.

In 1978 and 1979 three groups almost simultaneously published work demonstrating that transport of the large neutral amino acids across the blood-brain barrier was not uniformely altered. Zanchin and his colleagues in Padua (1979, I and II) carried out two studies, one <u>in vitro</u> and one <u>in vivo</u>, of the brain uptake of amino acids four weeks after PCS in the rat.

They showed that while neutral amino acid permeability was greatly increased (the most important increase being for TRP) the basic amino acids lysine and arginine had a greater than 50% decrease in transport. This observation that there was selective modification of transport of the different amino acid classes was an important one since it now became unlikely that non-specific factors such as variation in blood flow or changes in passive diffusion across the blood-brain barrier were operative. The other important observation was that there were regional differences in altered permeability. Thus while the decrease in cerebral permeability observed for basic amino acids led to a significant decrease of lysine and arginine concentrations in the cerebellum and pons-medulla, arginine values were increased by about 30% in the forebrain. This led to the concept that other local changes such as altered metabolism, protein synthesis, or transport systems other than those at the blood-brain barrier were involved in a complex process. The work of James et al (1978) was published just before that of Zanchin, and essentially showed the same results in portacavally shunted rats. The other paper published in 1978 from Fischer's group was that of Rosen et al. This study examined the effect of alteration of nutritional regimen (including intravenous amino acid infusion) in PCS and control animals. Dietary alterations produced no effect on the plasma amino acids or brain TYR of control animals. By contrast PCS animals had marked elevation of the plasma AAAs and of brain TYR, but these changes could be modified by infusion of amino acids to produce a positive nitrogen balance. Thus it became apparent that the level of general metabolic balance might be important in conditioning changes at the blood-brain interface.

In 1979 a further important thread was woven into the growing tapestry. James and his colleagues in Fischer's laboratory proposed a "unified theory of portal-systemic encephalopathy" in which it was suggested that GLN levels in the brain might modify the transport of neutral amino acids across the blood-brain barrier by an exchange mechanism. This was a potentially important concept since it re-introduced ammonia metabolism directly into the equation and took account of the possibility that the brain might control the delivery of amino acids and amines into its own metabolic environment by a feed-back mechanism.

Virtually all of these studies had been carried out upon whole brain, or supratentorial brain, or had been performed on tissue slices in vivo. Mans and her colleagues (1982) used a novel technique of quantitative autoradiography to measure the uptake of labelled amino acids and their clearance from the plasma in PCS rats seven weeks after operation. The rates of clearance of PHE, TRP and LEU from the plasma and uptake into the brain were increased by 80%, 200% and 30% respectively, while the clearance and uptake of the basic amino acid lysine was depressed by 70%. Rates of transport were altered unequally in different areas, with fluxes into the reticular formation, hippocampus and amygdala much greater than in other areas. Thus it became even more clear from this work that in the pathological state induced by PCS, it is not possible to predict the rate of influx purely on the basis of plasma amino acid concentrations, but that independent mechanisms influence transport of specific amino acids. In this instance the increase in transport was greater

for TRP than for PHE and greater for either than for LEU. These authors did not support the concept of James and his colleagues (1979) implicating GLN as an exchange molecule for neutral amino acids, largely because of the relatively weak transport affinity of the carrier for GLN, and because the GLN exchange theory could not readily be extended to take account of the transport of basic amino acids which had now been shown to be decreased after PCS.

Fischer's group had shown in shunted dogs (Fischer et al, 1976) and in man (Fischer et al, 1974) that manipulation of the plasma amino acids by infusion of specially formulated solutions could cause reversal of coma: these results will be further reviewed below in relation to clinical trials of amino acid therapy. In 1982 Rossi-Fanelli and his colleagues in Fischer's laboratory returned to a waking animal model, in the form of dogs with carotid loops and lateral ventricle cerebro-spinal fluid cannulae. They demonstrated that infusion of a mixture of PHE and TRP for several hours into the carotid artery of otherwise normal animals induced coma. These changes were associated with 32-fold increase of CSF TRP, 16-fold increase of PHE and a 9-fold increase of TYR, with doubling of CSF TRP, but without changes in CSF GLN or in plasma ammonia. Addition of BCAAs to the infusate prevented the onset of coma and also the increase in CSF octopamine. Not all the changes were abolished, however, since CSF levels of phenylethanolamine were increased to the same degree as in the animals without BCAAs, and there were 10-fold and 13-fold increases of PHE and TRP respectively. The authors forestalled the predictable criticism that these infusions resulted in concentrations many times higher than those seen in

spontaneous post-shunt hepatic encephalopathy. They repeated the experiment with only half the concentration of TRP in the infusate, and once again induced coma, with somewhat lower plasma and CSF TRP levels. They also commented that higher levels of TRP and other AAAs would be needed in this preparation to achieve CNS penetration, since this model lacks the reduced serum albumin and increased free fatty acids (both of which affect the availablity of TRP) seen in models with PCS or pre-existing liver dysfunction. Furthermore in this model the levels of BCAAs competing for the blood-brain barrier transport mechanism were normal. Moreover, the mechanism for acceleration of neutral amino acid transport in exchange for increased brain GLN which the same group had proposed (James et al, 1979) would not be operative in this model since the CSF GLN was normal. While these arguments are rational and may be correct it is not possible to prove the case in a concrete manner. The two possible mechanisms for these observations are that prevention of coma was achieved by competition for a common transport mechanism (the "classical" amino acid/false neurotransmitter theory), or alternatively that the BCAAs themselves might fulfil a critical metabolic role within the CNS, since unlike the aromatic amino acids they may be actively metabolized in the brain as an energy source. Despite the reservations discussed here this paper represents the first demonstration of a metabolic coma-like state with no increase in blood ammonia and an unchanged CSF GLN. It is strong supportive evidence of the importance of increased brain concentration of AAAs in relation to PSE.

Beaubernard et al (1983) showed that oral feeding of BCAAs

in PCS rats reduced the level of excitability and cortical awakening, as well as improving the turnover of cerebral TRP.

In 1982 the Padua group again made an important contribution to this complex field, when Rigotti and his colleagues showed that some of the plasma and brain amino acid abnormalities seen after PCS were prevented by concurrent hepatic arterialization. These workers demonstrated that arterialization could prevent the loss of body weight caused by PCS and maintain the relative liver weight at a normal level. The arterialized animals showed the same reduction in BCAAs as PCS rats, but showed a significantly smaller elevation in the plasma AAAs. PCS rats showed a large rise of GLN, citrulline and histidine and of the AAAs TYR, PHE and TRP in the brain. Arterialization produced higher levels of histidine and of AAAs than control animals, though they were decreased compared to PCS rats. The high brain GLN and citrulline levels were not modified by liver arterialization. These workers demonstrated that of the two principal agents proposed as the main cause of the increase in AAAs in the brain (decreased plasma levels of BCAAs or increased blood-brain transport of neutral amino acids) liver arterialization corrects the former but does not modify the latter. The measured brain uptake of all three AAAs was approximately doubled compared with control animals and there was no difference between PCS and arterialized rats. This result may be in favour of the GLN exchange hypothesis of James et al (1979), since plasma ammonia levels remained the same in both of these preparations, associated with increased brain GLN which might be involved in rapid exchange with the plasma neutral amino acids across the

blood-brain barrier.

In April of 1984 Fischer's group (Jonung et al, 1984) provided one further valuable piece of evidence in favour of the GLN exchange hypothesis. These workers measured the effect of 24 hours of ammonia infusion on brain amino acid levels in normal rats and rats pre-treated with methionine sulfoximine (MSO), an inhibitor of glutamine synthetase. The rats without MSO pre-treatment developed high plasma concentrations of GLN, whereas the MSO treated animals had low levels, presumably reflecting inhibition of glutamine synthetase in peripheral tissues. Brain levels of GLN and of large neutral amino acids rose in untreated rats, while those treated with MSO showed significantly lower brain levels of large neutral amino acids, only slightly higher than saline infused controls. Compared to rats not receiving MSO pre-treatment, MSO treated rats also had higher brain concentrations of the basic amino acids lysine and arginine. Thus pre-treatment with MSO blocked the ammonia-induced rise in brain GLN, but resulted in higher brain ammonia levels than untreated animals. Therefore the increase in brain levels of large neutral amino acids during ammonia infusion appears to be related to the rise in brain GLN concentration and not directly to the high brain ammonia concentration per se. The authors also note from unpublished observations that there is direct evidence for competition between GLN and PHE for the same transport system, although the affinity for GLN is relatively low and high levels are required to exercise important competition. Nevertheless it is possible that high brain concentrations of GLN might significantly impede the efflux of large neutral amino

acids from the brain. It is also possible that a concentration gradient of GLN (and other large neutral amino acids) from the brain to the blood might "drive" exchange transport for other large neutral amino acids. This effect (known as "transacceleration") may be the cause of the increased unidirectional blood to brain transport of large neutral amino acids which has been observed in rats after PCS. These authors discussed the possibility that this is a function of gammaglutamyl transpeptidase which is known to be present in cerebral capillaries. This paper is important evidence for a potential role for brain GLN in modifying blood-brain transport of large neutral amino acids in conditions of hyperammonaemia.

The above account represents the present complicated state of development of the amino acid transport story. While some of these observations appear to be progressing in a very promising direction it may still be fair to close this section with the words of Hal Conn, even though they were written before the developments of the last five years:

> "It is likely that these amino acid abnormalities represent foot prints in the shifting metabolic sands of PSE. Whether they lead directly to the cause of encephalopathy, point in the general direction of major encephalopathogenic derangements, or lead the investigator on a tortuous path to coincidental sequelae of independent, unrelated metabolic processes must await further research" (Conn and Lieberthal, 1979).

4.4 The blood-brain barrier

It has been known for more than half a century that the central nervous system does not take up intravenously

administered dyes, and it has been assumed that specific mechanisms exsist which regulate the micro-environment of the brain. These mechanisms are collectively referred to as the blood-brain barrier, so that while this barrier is anatomically sited at the capillary endothelial cell of the cerebral blood vessels, conceptually it is partly a physical and partly a metabolic barrier. For many compounds transport across the barrier is non-specific and depends upon their lipid solubility and diffusibility, whereas for certain compounds transport is facilitated by carrier systems. A number of these aspects have been considered above in relation to the transport of amino acids into the CNS. However, one early and important suggestion was that PSE is associated with a non-specific breakdown of the blood-brain barrier.

The principal evidence in favour of this view was presented by Livingstone et al in 1977, who showed that in acute hepatic failure produced by total hepatectomy in the rat diffuse staining of grey and white matter of the brain by Trypan blue was observed as well as increased penetration of some molecules that do not normally enter brain, such as inulin and L-glucose. However, the hepatectomy model is known to produce rapid onset of cerebral oedema with major histological changes but without the specific changes seen in light and electron microscopic studies following PCS (Zamora et al, 1973). Cangiano et al (1981) using serial CSF samples in man showed increased transport of neutral amino acids, especially in hepatic encephalopathy, but preferred the concept of a "leaky" blood-brain barrier to the GLN exchange mechanism dsecribed above. However, there is evidence that in PCS and

other PSE models there is actually decreased transport of some moieties (Cremer et al, 1977; Zanchin et al, 1979; James et al, 1978), strong evidence against a non-specific defect. Sarna et al (1977) have also shown no change in the net transport of radiolabelled sodium across the blood-brain barrier. Mans and her colleagues (1982), as already referred to above, demonstrated that permeability of the blood-brain barrier to lysine was greatly depressed in PCS rats. Horowitz et al (1983) confirmed that the difference in distribution of Trypan blue dye in Livingstone's work was due to a non-specific increase in permeability, but that transport of alpha-aminoisobutyric acid was highly selective, being only taken up by certain areas of grey matter. This discrepancy could be explained by differences in the transport properties of the two tracers. Trypan blue dye binds tightly to plasma proteins and will thus be distributed throughout the extracerebral fluid and move through the interstitial space by diffusion. By contrast, substances which are taken up by the brain cells will be trapped in the regions of the brain where they cross the blood-brain barrier. Essentially the blue dye in Livingstone's experiments was flowing with the stream of oedema fluid resulting from the hepatectomy preparation. Nevertheless, Horowitz's work lends partial support to Livingstone's suggestion that in acute liver failure there is a diffuse transfer of solutes into the CNS which may contribute to encephalopathy by compromising the homeostasis of cerebral fluid balance. Laursen (1982) on the other hand has demonstrated by quantitative microscopy that there are ultrastructural changes in astrocyte end-feet and processes which might result in diminution of blood-brain exchange at this site, and these

findings are in keeping with those of Zamora et al (1973) demonstrating both increased folding and an actual increase in basement membrane substance eight weeks after PCS. Thus the bulk of evidence is in favour of overall maintenance of the integrity of the blood-brain barrier, at least in models of chronic PSE. Nonetheless, there may be a diffuse, non-specific defect in the blood-brain barrier in some models of <u>acute</u> liver failure.

4.5 Clinical efficacy of branched chain amino acids

Early evidence from Fischer's laboratory (Fischer et al, 1974) showed that infusion of a branched chain enriched amino acid solution into patients with acute hepatic encephalopathy would restore to normal the diminished ratio of BCAA to AAA, improving the encephalopathy in 8/8 patients with cirrhosis, and 1/3 patients with fulminant hepatitis. There appeared to be a dose response curve between the amount of solution infused and the lowering of plasma AAA and ammonia. Conclusions regarding improvement of encephalopathy were indefinite, but it had undoubtedly proved possible by use of an appropriately formulated solution to administer a greater nitrogen load than would have been possible in patients with severe liver disease using unbalanced solutions. Similar results were advanced in dogs and in rats, and these have been discussed above.

Since that study numerous uncontrolled case reports have appeared in the literature on branched chain amino acid administration to cirrhotic patients with encephalopathy: these will not be described in detail here, but comment on these anecdotal reports (e.g. Freund et al, 1982) is made in the review by Sax et al (1986). However, it is only in the light of the prospective controlled clinical trials which have now become available that it is possible to evaluate branched chain amino acid therapy.

There have now been seven controlled trials of intravenous BCAA therapy, and several of oral treatment. The key features of these studies are summarized in Table 6.2. It can be seen that

Table 6.2 Trials of branched chain amino acid therapy

Authors	Patients	n	Treatment	Control	Outcome
ORAL					
Erikssen 1982	Chronic PSE	7x	BCAA	Sucrose	No difference
Keohane 1983	Grade 1-3	10	BCAA-enriched	Uncontrolled	+ve N balance
Sieg 1983	Chronic PSE	14	BCAA-enriched	Uncontrolled	No effect
Watanabe 1983	Cirrhotic	6	BCAA-enriched	Uncontrolled	NCT time imp.
Horst 1984	Chronic PSE	37	BCAA	Normal protein	Less PSE
Egberts 1984	Minor PSE	22	BCAA	Casein	Less PSE. Better N balance
INTRAVENOUS					
Rossi-Fanelli 1982	Severe PSE	34	BCAA+20%D	Lactulose+20%D	No difference
Strauss 1983	Grade 1-3	29	BCAA	Neomycin	Shortened coma
Wahren 1983	Grade 2-4	50	BCAA+D+lipid+ lactulose	D+lipid+ lactulose	No difference
Gluud 1983	Grade 2-4	20	BCAA+20%D	20%D	Possible imp.
Freeman 1983	Cirrhotic	17	BCAA+lactulose	5%D+lactulose	No difference
Michel 1984	Grade 2-4	47	BCAA+glu+lipid	Standard AA+glu +lipid	No difference
Cerra 1983	Acute PSE	22	BCAA+20%D	20%D+neomycin	Improved on BCAA
Cerra 1985 (MC)	Acute PSE	81	BCAA+20%D+ neomycin	20%D+ neomycin	Improved on BCAA
Fiaccadori 1985	Acute PSE	48	BCAA/BCAA+Lactulose/Lactulose		100%/94%/62% rec
Cangiano 1986	Grade 3-4	14	BCAA+20%D	(uncontrolled)	10/14 recovered CSF Glu fell

MC = US Multicentre Trial
x = crossover study
20%D = 20% Dextrose/water

the results are far from unanimous, and this is partly due to the small numbers of patients in most of the studies, and partly to the widely varying trial designs. It is only in the multicentre studies that adequate numbers have yet been accrued to ensure freedom from bias and from Type II statistical error. Moreover, the aetiology of the liver disease and the proximate cause of the encephalopathy differ considerably amongst the trials: for example the percentage of alcoholics included varies from 30% (Rossi-Fanelli et al, 1982) to 100% (Gluud et al 1983). This may be very important since the outcome of alcoholic cirrhosis is significantly influenced by the recent alcohol consumption. In two trials (Rossi-Fanelli; Wahren) bleeding and infection were the major precipitating factors, while in Cerra's study many patients had PSE as a result of operative trauma. A further factor is the grade of encephalopathy on entry to the trial, since patients in Grade II coma have a high rate of spontaneous recovery; conversely the mortality for Grade IV coma is high whatever treatment is used. For this reason some workers excluded patients until they had been in coma for at least 24 hours (Wahren) or had failed to respond to conventional therapy for 48 hours (Cerra et al, 1983).

Further, the therapy used in the trials also differed widely: in the (negative) European trial (Wahren) the treatment solution contained only the three BCAAs, while that used in the (positive) U.S. Multicentre Trial (Cerra et al 1985) also contained other essential and non-essential amino acids. On theoretical grounds the balanced solution is preferable, since a solution containing only BCAAs would impede entry of other large

neutral amino acids to the brain and possibly deprive it of essential amino acids.

The third important variable amongst these studies is the nature of the control treatment, and the use of concurrent treatments, nutritional and otherwise. It will be evident from Table 6.2 that not all patients had equal access to conventional therapies such as lactulose, and also that the degree and nature of the non amino acid nutritional support differs between groups. Importantly, two "negative" studies (Wahren; Michel) used lipid infusion as part of the regimen: since fat emulsion displaces tryptophan from binding sites on albumin there may have been a tendency to higher plasma free tryptophan in these patients. Conversely, in those patients treated without fat but with glucose and insulin the plasma free tryptophan will have been reduced (Miyai et al, 1985), a further factor in favour of recovery.

In relation to the mechanisms of PSE, interesting peripheral results have appeared from some of these therapeutic studies. Riggio and his co-workers (from the same group as Rossi-Fanelli) showed that infusion of BCAA in patients in Grade III-IV coma lowered the CSF concentrations of octopamine and phenylethanolamine, in parallel with recovery of neurological function. Paradoxically, however, after full neurological recovery the CSF levels of these false neurotransmitters had risen again to pre-treatment values - further conflicting evidence regarding the primacy of false neurotransmitters in PSE.

As regards the value of oral BCAA therapy, there is a suggestion that BC-enriched oral mixtures may be superior to conventional protein for enteral support in chronic PSE (Table 6.2). Clearly this therapy is unsuited for patients in deep coma.

Whatever the evidence in relation to the role of BCAA therapy in securing arousal from coma, there appears to be little doubt that when nutritional support is indicated in these patients a higher level of nitrogen administration and greater positive nitrogen balance can be safely achieved with BCAA or BCAA-enriched solutions than with other solutions, and it may be for this reason that these solutions eventually become part of routine therapy. In the meantime, further trial results must be awaited before the use of this expensive modality is accepted as clearly superior to conventional management, and the results to date offer no conclusive evidence in relation to the relevance of amino acid imbalance in the pathogenesis of PSE.

5. CNS CHANGES IN PSE

5.1 Structural changes in animal experiments

The present studies have demonstrated that the characteristic Alzheimer II changes seen in a high proportion of PCS animals 5 and 10 weeks after operation occurred to a significantly lesser degree after PCT. These changes were shown first in the dentate nucleus, where the differences shown were relatively small, and subsequently in the Bergmann astrocytes of the cerebellum in which there was a very marked difference.

The findings after PCS are in keeping with previously published work, probably best summarized by the studies of Cavanagh's group (Zamora et al, 1973; Cavanagh et al, 1972). The detailed electron microscopic studies from Cavanagh's group showed changes identifiable as early as 5 weeks in the Bergmann glia, with increased density of endoplasmic reticulum and of mitochondria in the cytoplasm followed by diffuse swelling of the astrocyte processes and end-feet with morphological evidence suggesting increased metabolic activity in the peri-nuclear region of these cells. Diemer (1976) also observed changes in the Bergmann astrocytes following carbon tetrachloride induced liver damage in rats, with an absolute increase of up to 20% in the Bergmann cell numbers. Those animals with the longest duration of administration of carbon tetrachloride also showed the most marked lobulation of the Bergmann nuclei. The Bergmann cells allow a useful area for study, since they occur as a well-defined layer with one of the highest glial to neuronal ratios in the body (Diemer, 1976). Lahl (1974) has demonstrated

not only morphological astrocyte changes, but also the suggestion that Bergmann cell numbers are increased following PCS and carbon tetrachloride treatment, possibly due to glial transformation from pleuripotent microglial cells.

Only one group has produced important confirmatory evidence in support of the results presented here demonstrating the importance of liver dysfunction as well as portal-systemic shunting in the production of CNS changes. Vassanelli et al (1983) demonstrated that either PCT or hepatic arterialization following PCS produced marked reduction of the lesions in the cerebellum, bulb and pons produced by PCS alone. It has already been noted that this work is not only in keeping with our own results, but also accords well with those of Castaing et al (1982), who demonstrated that in the absence of liver atrophy due to decreased hepatic blood flow, portal-systemic shunting did not result in post-shunt encephalopathy, as measured by changes in cortical awakening.

5.2 Metabolic importance of neuroglia

We have already reviewed the hypotheses regarding glial cell function. In summary, the weight of evidence suggests that the astrocytes, which are anatomically orientated in close relationship to the synaptic junctions (Peters and Palay, 1965), act in part as accessory metabolic units for the neurones, and in part as regulators of neurotransmitter metabolism. It is relevant here to review again the role of the neuroglia in the uptake, inactivation and re-cycling of neurotransmitter substances.

The importance of amino acids as CNS neurotransmitters has become recognized in the last 10-15 years (Baldessarini and Karobath, 1973). A list of the principal putative neurotransmitters is shown in Table 6.1. The neutral amino acids GABA, glycine, taurine and alanine have an inhibitory effect on central neurones, while the acidic amino acids GLU and aspartate have potent widespread stimulatory effects. An important functional property of the synapse is the ability to provide spacial and functional specificity for specific neurotransmitters, including the amino acids. One possible mechanism is the selective synthesis of specific amino acids in the neurones, but this has not been shown to occur for the amino acid neurotransmitters. The currently favoured mechanism is that of selective uptake and release of pre-synthesized amino acids from the pre-synaptic terminals. "Low affinity" uptake of amino acids into the neurones has been demonstrated, and this may be the most important route of uptake for GABA. However, several amino acids, including GLU, are principally taken up by more specific "high affinity" receptors. Moreover, Currie and Kelly (1981) have shown by autoradiographic studies that the high affinity uptake of glutamate is solely a prperty of the glial cells, in contrast to the neurone-specific uptake of GABA into GABAergic neurones. This means of inactivating and conserving neurotransmitter substrates following their release at the synapse appears to be related to areas of specific affinity localized to different brain regions and to different cell layers, and the glial cells appear to be particularly important in this respect.

With the exception of acetylcholinesterase none of the enzymes involved in the degradation of neurotransmitter molecules is to be found in the pre- or post-synaptic neural membranes. By contrast, GABA transaminase and monoamine oxidase are mitochondrial enzymes which are found in the astrocytes, and catechol-o-methyltransferase is a ubiquitous soluble enzyme also shown to be localized in these cells. Glutamine synthetase has been shown by histochemical techniques to be located entirely in the glia (Martinez-Hernandez et al, 1977), and inhibition of this enzyme by MSO leads to convulsions in experimental animals. The key role for this enzyme in the metabolic re-cycling of glutamate as an excitatory CNS neurotransmitter is shown in Figure 6.3. This is in accord with the finding by Henn and Hamberger (1971) that the glial cell fraction takes up 20 times more glutamic acid than does the neuronal cell fraction of brain extracts. There is now increasing support for the concept of a neuronal-glial cycle in which GLU passes from neurones to glia and is returned to the neurones as GLN. The efficiency of this as a homeostatic mechanism is indicated by the fact that the total concentration of the three putative transmitter amino acids GLU, aspartate and GABA, which comprise along with the physiologically inactive GLN 70% of the free amino acids in the brain, remains constant under most conditions, although individual levels may vary widely (Currie and Kelly, 1981). Moreover, there is also a striking constancy of the total nitrogen content of the sum of tissue ammonia, GLU, GLN, GABA and aspartate. The synthesis of GLN from GLU is the main pathway for ammonia detoxification in brain, so that this neuronal-glial cycle also provides a mechanism for regulation of tissue ammonia levels.

To understand the importance of small changes in local concentrations of GLU, GLN and other neuroactive substances it is necessary to review the topic of metabolic compartmentation in the central nervous system.

5.3 Metabolic compartmentation in the CNS

The demonstration of division of GLN and GLU metabolism in the CNS into several distinct compartments largely derives from the evidence of Berl and his colleague (Berl et al, 1962; 1969; Berl, 1971: collected reviews in Berl and Clark, 1975). These workers examined the handling of radiolabelled ammonium acetate infused into the cat carotid artery. Most of the nitrogen label appeared in GLN in the brain, and in urea in the liver and the blood, highlighting these two distinct metabolic pathways for ammonia detoxication in liver and brain. The brain GLN levels increased without a corresponding fall in GLU, consistent with the high levels of glutamine synthetase already noted in the brain. However, the brain GLN labelling was found to be between 4 and 13 times greater than labelling of GLU, suggesting that cerebral GLN is derived from a subcompartment of GLU which does not exchange readily with other tissue GLU. These dynamics characterize the "small rapidly metabolized" GLU pool which is preferentially used for GLN synthesis in the brain. The estimated half-lives of the GLU compartments showed that the small rapidly metabolized pool in the brain had a half-life of less than one hour, compared with a half-life of six to eight hours for the large slowly metabolized pool. The corresponding half-life of free GLU in the liver was 20-30 minutes, representing a rapidly metabolized compartment for conversion of

GLU to aspartate for urea formation. Other workers (Barkulis et al, 1960) have estimated that less than one third of the total brain GLU is in the small active pool, and that this pool is in very slow equilibrium with the large total pool. Garfinkel (1966) estimated from computer modelling of these systems that the small active pool might constitute between 14 and 19% of the total GLU pool. These pools of course are not entirely separate: there is a slow "leak" between the two.

Extrapolating from the evidence for metabolic compartmentation to the localization of different metabolic pools in known cellular structures is a major step, but there is now some evidence suggesting identification of the small metabolic pool with astrocytes. The location of glutamine synthetase in astrocytes has already been noted, and in these cells this enzyme is found in the mitochondria and in the microsomes. Glutamate dehydrogenase is also found in mitochondria, and there is evidence that freshly synthesized GLU is aminated in mitochondria before mixing with other tissue pools of GLU, while exogenously administered GLU is aminated in the endoplasmic reticulum (Berl, 1971). Zamora et al (1973) have suggested that the observed increase in size of the small metabolic compartments six weeks after PCS is the metabolic parallel of the morphologic increase in astrocyte size observed, confirming that the astrocytes are the anatomical site of the small GLU and GLN compartments. Other workers have also supported the localization of the small metabolic pool in the astrocytes since GLN has been shown pricipally to be a glial metabolite in the CNS (Williams et al, 1972; Benjamin and Quastel, 1972).

The existence of discrete metabolic pools for GLN/GLU metabolism may explain the conflicting results reported by Dennis and Clarke (1977), suggesting that a major route for disposal of brain GLU is by transamination to aspartate rather than by GLN synthesis: this may be a major metabolic route in the large pool, while the small pool uses principally the GLN synthetase route for GLU metabolism. This would also be in keeping with the suggestion that there is functional heterogeneity in the subpopulation of mitochondria which can be sedimented from brain extract (see Berl, 1971). Finally it must be pointed out that it has also been suggested that there is compartmentalization of other substances, including alpha-ketoglutarate: this appears to be contained in at least two pools, one of which has a very high specific activity and is associated with rapid GLN formation (Berl, 1971).

The presence of co-existent metabolic pools of independent activity and of different size is important in the interpretation of minor changes in GLN and GLU observed in brain tissue extracts. Changes in overall concentrations of neurotransmitters and other metabolites in brain may be of limited relevance to the pathogenesis of PSE (Jones, 1983). On the other hand, minor changes in the small metabolically active neurotransmitter pools may not be reflected in gross changes in brain tissue level, which represent a summation of both the small and large metabolic pools, of which total the small pool may constitute as little as 14%. Nevertheless, such small changes at the crucial neuronal-glial interface may have major functional effects. It has been long recognized that CSF levels of GLN correlate better

with the degree of hepatic coma than ammonia levels (Gilon, 1959), and this has been observed in a several of the experimental studies discussed above.

Changes in tissue levels of GLN and GLU following PCS and in other models of PSE have been seen. Williams and his colleagues (1972) showed a 2.4 fold increase in brain GLN in the cerebrum and in the cerebellum and brain stem six weeks after PCS, but no significant change in GLU. Funovics and Fischer (1978) showed that following PCS and hepatic artery ligation brain GLU was diminished by 20% while GLN was increased by a factor of 3.5 and ammonia by a factor of 4.5. Free ammonia directly inhibits utilization of GLN for GLU formation, and ammonia infusion in normal rats led to a gradual development of a basal eflux of GLU from a pool which was tentatively ascribed to glial cells (Hamberger et al, 1980). In PCS rats (which have been subject to a prolonged environment of increased ammonia) this response is lost, suggesting that ammonia may be a regulating factor for the biosynthesis of transmitter GLU. It has been suggested that one mechanism for encephalopathy in the presence of an imbalance between GLN and GLU is that GLN is taken up by nerve terminals in competition with GLU and released by depolarizing stimuli, thus acting as a "false neurotransmitter", since GLN is neuropharmacologically inert (Baldessarini and Yorke, 1974).

Examination of the levels of GLN, GLU and ammonia in the present studies show no statistically significant differences. However, in view of the existence of compartmentalization it may be relevant to speculate on the possible significance of small changes and trends (Figures 5.16 and 5.17). Values of GLN in the

brain stem and cerebellum in PCS animals were higher (not statistically so) than those in control and PCT animals, and levels of GLU in the cerebrum and cerebellum showed a statistically marginal degree of reduction. The direction of these changes is consistent with the concept that small pool GLN synthesis from GLU is increased in this model, or that re-cycling of GLN to GLU is deficient. The relevant pathways are illustrated in Figure 6.3. It is possible to view the astrocyte in the light of all the previous evidence as a "GLU pump" with the function of re-cycling excitatory neurotransmitter for uptake by the neurones. A block in the conversion of GLN to GLU for neuronal uptake might lead to an increase of small pool GLN, with little increase in total brain GLN. This lack of effect on overall brain levels may be emphasized by the fact that changes are localized even within brain regions. Freeze-blow techniques for rapid extraction of the entire supratentorial brain might be expected to miss small changes, but even division of the brain into subregions such as performed in this work might fail to reveal minor but important changes in key sites of the nervous system. In these localized areas GLU might fall as a result of failure of GLN recycling in the metabolically active neurotransmitter pool. This would ultimately lead to a degree of excitatory neurotransmitter failure. Moreover, loss of the GLN synthetase step of GLU metabolism might result in a local increase in ammonia concentrations, which may exert local toxic effects. Thus there may be a <u>local</u> increase in ammonia activity superimposed on the already present background of elevated plasma and brain ammonia levels as a result of portal-systemic shunting.

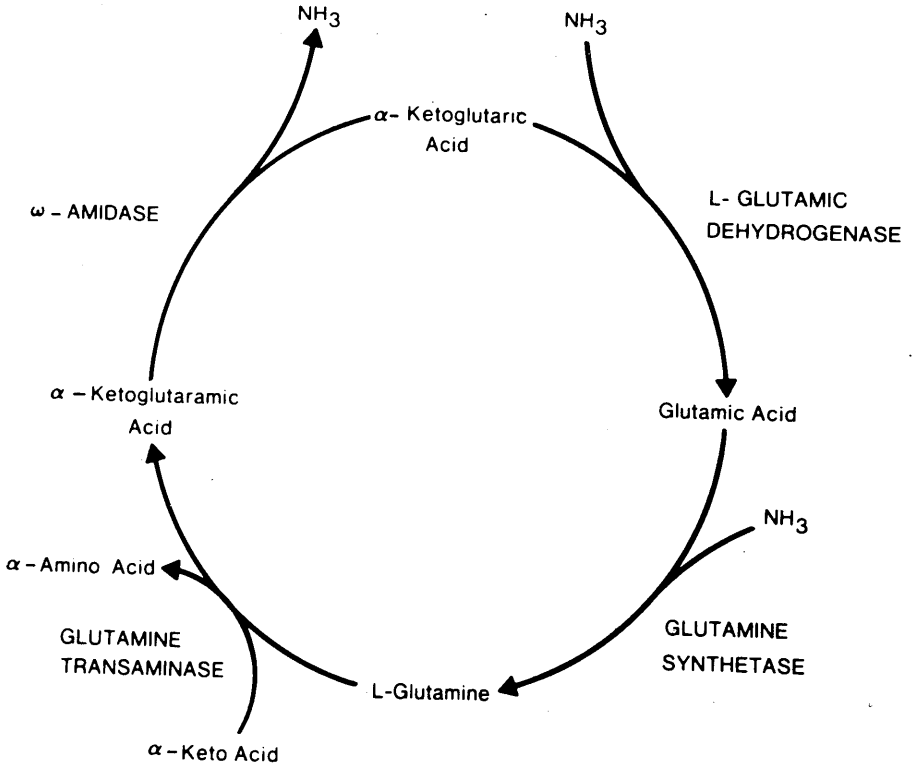


Figure 6.3

The metabolism of ammonia, glutamate and glutamine.
Note that the enzyme glutamine synthetase is localized in the glia, and that this may be the major site for uptake and inactivation of neurotransmitter glutamate.

Other effects of increased local GLN concentrations might be postulated. It has already been noted that GLN and ammonia may accelerate large neutral amino acid transport across the blood-brain barrier. These changes may to some extent exercise negative feedback autoregulation of entry of the already excessive aromatic amino acid neurotransmitter precursors into the brain. However, this compensatory mechanism might be rapidly overwhelmed, and indeed the local changes described here may be insufficient to prevent a more global increase in the uptake of these amino acids in the brain as a whole, which may be followed by diffusion to critical metabolic areas. Another possibility is the diversion of GLN to alpha-ketoglutaramate by GLN transaminase (see Figure 6.3). Alpha-ketoglutaramate has been found to be increased 4-10 fold in the CSF of patients with hepatic encephalopathy, and to cause EEG changes similar to those of hepatic coma when infused into the lateral cerebral ventricles of normal rats (Vergara et al, 1974).

It should be noted that this hypothesis of astrocyte "glutamate pump" failure does not postulate any specific agent as being primarily responsible for the syndrome. This could result from a generalized failure of cerebral oxidative metabolism, which some workers have reported in experimental hepatic failure (Hindfeld and Siesjo, 1971; Holmin et al, 1983), or localized areas of altered metabolism in the astrocytes. In particular, it is not necessary to postulate that ammonia, amino acids, or any of the other moieties advanced as the possible primary cause of PSE be the mechanism by which astrocyte metabolism is rendered abnormal. This could be due to any of these molecules, or to

synergism amongst them, or to a factor as yet unidentified, with the same ultimate effects on CNS neurotransmission resulting from failure of the astrocyte "support unit". This type of approach is largely in keeping with the view put forward by Diemer (1976) of PSE as a "glial syndrome".

6. CONCLUSIONS AND FUTURE PROSPECTS

The results in this Thesis confirm the major importance of maintenance of hepatic mass and hepatic function in prevention of the CNS consequences of portal diversion. It is perhaps surprising in view of the now well-recognized role of portal hepatotrophic factors that perfusion of the liver by systemic blood is effective in producing this effect. However, the results of portacaval transposition in this Thesis, the recent abstract of Vassanelli and his colleagues (1983), and the results of hepatic arterialization presented by Castaing et al (1982) demonstrate this efficacy. These findings are brought into clinical relevance by the studies of Burchell (1976) showing that an increment of hepatic arterial flow following portacaval shunting was effective in reducing the incidence of PSE. The basis on which selective shunting, introduced by Warren in 1967, reduces the incidence of PSE rests principally on the continued perfusion of the liver by portal blood following the procedure. It is not clear why these shunts should continue to have a long-term advantage, since it has been well demonstrated that there is a gradual reduction in hepatopetal flow with the passage of time (Maillard et al, 1979). The metabolic adjustments which take place during such a slow reduction in hepatic flow are unknown, but may be sufficient to compensate for portal deprivation in a way which cannot be achieved with an abrupt total shunt. Thus by the use of haemodynamic measurements and selective shunting we may be closer to Conn's objective: "we must learn to select better the patients we shunt or to shunt better those whom we select" (Conn, 1973).

As to the pathogenesis of PSE, recent research has demonstrated many subtle derangements, and examination of the transport of amino acids and amines across the blood-brain barrier has revealed a great deal of precise metabolic information about the CNS changes in various animal models. Our own studies showing a difference in the amino acid profiles of rats treated by transposition and by total shunt, but it remains by no means certain that the amino acid/false neurotransmitter hypothesis will provide the whole answer to encephalopathy. There remains, however, considerable evidence for an important role of astrocytes in this process, and it may be that further histochemical studies of these cells may add more to our understanding.

The model devised by Geiger in 1954, which established firmly in vitro a relationship between cerebral and hepatic metabolism, has not yet been repeated, confirmed or extended. It would seem that this type of perfusion experiment might help to unlock some more of the secrets of PSE, and to this end we have established a combined isolated perfused rat brain and liver preparation in our own laboratory.

It is apposite to conclude this work by quoting two eminent hepatologists, whose statements span almost 20 years:

"In the 83 years since it was first reported the Eck fistula has been reasonably successful in hiding its secrets as well as giving rise to many additional questions fundamental to an understanding of the functions of the intestine, liver and brain" (Bollman, 1961).

"When one has finished reviewing all of the postulated mechanisms for the biochemical pathogenesis of PSE, one is left with no completely satisfying answer and a number of quite unsatisfactory ones" (Conn and Lieberthal, 1979).

I believe that use of the portacaval transposition model, while not providing further definitive answers, has added an extra and valuable tool to the armamentarium of those who would unravel this complex syndrome.

APPENDICES

APPENDIX A: LABORATORY METHODS

(1) Nitrogen determination by Kjeldahl method

Principle: manual digestion of nitrogen, followed by ammonia estimation.

Digestion

To each Kjeldahl digestion flask add:
- 2 Kjeldahl tablets (1.2G pot. sulphate +0.05G mercuric oxide
- 2 glass beads
- 3ml conc sulphuric acid
- quantity of test material containing 4-20mg nitrogen (for urine, 5ml aliquot of 250ml diluted washings)

Digestion flasks placed at 30 ^{o} angle on digestion rack:
- 4 samples
- 1 blank (contains 5ml dist water in place of sample)
- 1 standard (1ml urea solution of 10mg/ml nitrogen)

Heat gently for 5-10 mins in fume cupboard to boil off excess water. Increase heat to vigorous boiling for 15-20 mins: when digestion is complete a ring of white precipitate forms around the neck of the flask. If frothing occurs, quickly remove flask and add 2-5 drops hydrogen peroxide, shake gently and replace on rack. After completion leave to cool for30 mins.

Wash contents out thoroughly with distilled water into 100ml volumetric flasks and make up to 100ml with distilled water: mix thoroughly before removing sample for analysis. Samples may be kept at 4 ^{o} C for up to 1 week before analysis.

Digest samples are a 1:200 dilution of original sample and should therefore contain 20-100ugms/ml nitrogen digest (standard 50ugm/ml).

Analysis

Ammonia estimation by autoanalyzer.
Ammonium sulphate standard (200ugm nitrogen/ml stock diluted to give standard range of 10-120ugm nitrogen/ml).

Results of autoanalyzer output checked for zero blank and value of 50ugm/ml standard digest. If incorrect, this suggests either nitrogen contamination or loss, and all digestions should be repeated.

Urea standard: 2.143G urea/100ml = 10mg nitrogen/ml standard.

For nitrogen in rat diet 41B, powdered, use 0.25G and 6ml conc sulphuric acid + Kjeldahl tab + glass beads. DO NOT HEAT!!
Add 4-6ml hydrogen peroxide. Solution will froth and then clear. Heat until digested as above.

(2) Glutamate and glutamine assay

Reference
Bergmeyer, 1974

Principle

(1) $\text{L-glutamine} + H_2O \xrightarrow{\text{glutaminase}} \text{L-glutamate} + NH_3$

(2) $\text{L-glutamate} + H_2O + NAD^+ \xrightarrow{\text{glutamate dehydrogenase}} \text{2-oxoglutarate} + NADH^+ + NH_4$

Reaction (1) is allowed to reach completion before a portion of the reaction mixture is taken for glutamate assay (2). A second aliquot of the blood or tissue extract is assayed for glutamate only (2), and the glutamine concentration obtained by the difference.

Reagents for (1):

1. Acetate buffer: 0.5M, pH 5.0
   a. dissolve 6.8 g $CH_3COONa\text{-}3H_2O$ in d/w and make up to 100ml.
   b. dilute 2.9 ml acetic acid (99% w/v) to 100ml with d/w.
   Mix 67.8 ml (a) with 32.2 ml (b).
   Store at 4 °C.

2. L-glutamine standard solution : 10µmol/ml.
   Dissolve 14.6 mg L-glutamine in d/w and make up to 10ml.
   Store at -15 °C.

3. Glutaminase: 24U/mg (Sigma Chemical Co).
   Store dessicated at -15 °C.
   Dissolve 1.0mg in 10ml acetate buffer.
   Store aliquots at -15 °C.

Reagents for (2)

1. Tris hydrazine buffer: 0.1M, pH 9.0
   To 1.5ml hydrazine hydrate add 15ml 1N HCl to pH 9.0
   Add 3ml 0.1M EDTA sol.
   Make up to 33.25ml with Tris HCl buffer pH 9.0, 0.1M.
   (Tris HCl buffer: 100ml 0.2M Tris; add 1N HCl to pH 9; make up to 200ml with d/w; prepare fresh.)
2. NAD^+ 2%
   20mg NAD + 1ml d/w. Prepare fresh.
3. ADP: 0.1M. Store at -15 °C. Prepare fresh every 2 weeks.
   47.12mg + 1ml d/w.
4. Reagent mixture: Tris hydrazine buffer 66.50 ml
   NAD 2% 3.50 ml
   ADP 0.1M 0.35 ml

   70.35 ml

5. Glutamate dehydrogenase: 10mg protein/ml in 50% glycerol
(Boehringer Mannheim)

6. Glutamate standard solution: 10 µmol/ml
16.91mg made up to 10ml with d/w. Store at -15 ° C.
Dilute 1:10 before use

Step (1)

Pipette into polypropylene tubes with caps:
acetate buffer 0.5ml
glutaminase 0.1ml
sample 0.4ml

1.0ml

Use 0.05ml standard and make up to 1ml with d/w
For blank use 0.4ml d/w.

Incubate at 37 ° C for 2 hours, cool in ice.
Use 0.1ml for glutamate assay.

Step (2)

Pipette into cuvettes:
(a) Hydrolysis samples:
Reagent mix 2.00ml
Sample from (1) 0.10ml
Mix, read E1 at 340 nm against water.
Glutamate dehydrogenase 0.02ml
Mix, read E2 after 30 mins.

(b) Glutamate only samples
Reagent mix 2.00ml
Tissue extract 0.05ml
Water 0.05ml
Mix, read E1 at 340 nm.
Glutamate dehydrogenase 0.02ml
Mix, read E2 after 30 mins, and repeat until no further increase in absorption.
For blank use 0.10ml d/w.
For glutamate standard use 0.05ml standard+ 0.05ml d/w

Glutamine content=total glutamate after hydrolysis (a) - glutamate only (b).

Ammonia assay

Reference:
Folbergrova et al, 1969

Principle:

$$NH^4_+ + \alpha\text{-ketoglutarate} + NADH \xrightarrow{\text{glutamate dehydrogenase}} \text{glutamate} + NAD^+ + H_2O.$$

Excess α-ketoglutarate and enzyme shift equilibrium to right.

Reagents

Use fresh deionized water.

1. Tris HCl buffer 0.2M, pH 8.1
   Make 100ml 0.4M Tris (4.85 G to 100ml d/w)
   Use 10N HCl to pH 8.1
   Make up to 200ml with d/w
   Store at 4 ^{o} C.

2. NADH solution 5mM
   3.5 mg + 1ml d/w
   Make up fresh.

3. Glutamate dehydrogenase
   50% in glycerol (Boehringer Mannheim)
   Spin at 13,000 g to clarify if necessary.
   Store at 4 ^{o} C.

4. Ammonia standard: ammonium chloride 5.3mg/100ml
   = 1umol/ml
   Make up fresh.

5. Reagent blank: neutralize 4ml 8% PCA with KOH/K_2CO_3
   and spin as for tissue extraction.
   Use supernatant for blank sample.

Reagent mix:

Tris HCl buffer 0.2M	20ml
α-ketoglutarate	50mg
ADP	100mg
NADH 5 mM	0.8ml
Water	18ml
	39ml

Make up fresh

Procedure

Into cuvettes pipette:

Reagent mix 1.5ml
Sample or blank 0.8ml (less if high levels expected)
Mix, read E1 at 340 nm against air
Glutamate dehydrogenase 0.04ml
Mix, read E2 at 340 nm after 30 mins, until no further decrease in absorption.

For standard use 0.05ml standard + 0.75ml d/w
Use separate blank of d/w to calculate the standard

Note:
Great care must be taken to prevent ammonia contamination of water used in all reagents. De-ionized water must be used, and care taken in the cleaning of glassware. A 'creep' on reading E2 indicates that fresh enzyme is needed.

(3) Tryptophan assay

Reference

Eccleston, 1973

Principle

Free tryptophan is condensed with formaldehyde and oxidized by ferric chloride in acid conditions to form a highly fluorescent substance called norharman. The norharman produced by the sample is measured fluorimetrically and the concentration determined by comparison with standards.

Reagents

1. 10% perchloric acid (PCA)
2. 30% PCA containing 4.5×10^{-4} ferric chloride
   12.16 mg hydrated ferric chloride (0.0073 mg anhydrous) to 100ml PCA.
   Store at 4 °C.
3. 1.8% HCHO solution
   2.37 ml 28% HCHO to 50 ml with d/w
   Store at 4 °C, make fresh every 2 weeks
4. 0.1M NH_4OH
   1.36ml 25% solution to 200ml d/w.
   Stable.
5. Tryptophan standard solution 250 nmol/ml
   5.1 mg tryptophan to 100ml 0.1M NH_4OH
   Make fresh every day.
   For range of standards dilute stock solution with 0.1M NH_4OH to obtain 50,100,200,250 nmol/ml.
6. Norharman standard solution (if required) 250 nmol/ml.
   4.12 mg norharman to 100ml 0.25% PCA.
   Make fresh.

Procedure

Separation of unbound tryptophan in plasma

Use Amicon 24-CF 50 centriflo cones (50,000 MW cut-off) with supports in conical 50ml centrifuge tubes.

1. Soak cones in d/w for 1 hour before use and centrifuge at 800g for 15 minutes to remove excess water.
2. Place 0.5 ml fresh plasma in cones and centrifuge at 800g for 15minutes at 20-22 °C.
3. Assay unbound tryptophan in ultrafiltrate (clear solution).

Notes

1. The binding of tryptophan to albumin is believed to be temperature dependent, so that all steps in the separation of unbound tryptophan are carried out at room temperature.

2. Plasma used must be fresh; if frozen, the binding to albumin will be altered. After separation, the ultrafiltrate and plasma for tryptophan determination may be frozen.

3. The time for centrifuging may be varied to yield more or less ultrafiltrate. Do not exceed 1000g when spinning cones or membrane will be damaged.

4. Rinse cones in d/w, allow to stand overnight containing NaCl 5%, rinse in d/w and store in 10% ethanol. Cones can be re-used about 10 times, or until protein passes through (ultrafiltrate becomes coloured).

Assay of tryptophan

1. Into glass culture tubes with screw caps pipette:

	standard	u/f sample	brain sample
Distilled water	1.80 ml	1.80 ml	1.30 ml
Standard solution	0.30 ml	---	---
Ultrafiltrate	---	0.06 ml	---
Brain extract	---	---	0.50 ml
PCA/$FeCl_3$ solution	0.90 ml	0.90 ml	0.90 ml
HCHO solution	0.30 ml	0.30 ml	0.30 ml

For total tryptophan in plasma use polyethylene centrifuge tubes (17.5 ml capacity)

Add	d/w	1.80 ml
	Plasma	0.03 ml
	PCA/$FeCl_3$	0.90 ml

Allow to stand for 10 minutes.
Centrifuge at 13,000g for 10 minutes.
Decant into glass screw-capped tubes with 0.13ml HCHO solution.

2. Mix all tubes well and incubate in hot air oven at 105 ^{o} C for 3 hours.
3. Allow to cool, decant into graduated tubes.
4. Make up to 3ml with 10% PCA solution.
5. Read fluorescence at 373 nm excitation maximum and 452 emission maximum.
6. Use standard curve to read off unknown concentrations.

APPENDIX B.

Detailed tables of results

Table B.1 Body weight changes in chronic studies in Cape Town.

Table B.2 Levels of ALT, LDH, CK and alkaline phosphatase up to 24 hours after control operation, PCT, PCS, laparotomy (LAP), anterior portal vein libation (PVL), hepatic artery ligation (HAL), and partial hepatectomy (PH).

Table B.3 AST levels up to 5 weeks after operation (Glasgow series).

Table B.4 ALT levels up to 5 weeks after operation (Glasgow series).

Table B.5 Alkaline phosphatase levels up to 5 weeks after operation (Glasgow series).

Table B.6 AST levels 1-10 weeks after operation (Cape Town series).

Table B.7 Albumin levels (as % of pre-op values) 1-5 weeks after operation (Glasgow series).

Table B.8 Slow α_1-globulin levels (as % of pre-op values) 1-5 weeks after operation (Glasgow series).

Table B.9 IgG levels (as % of pre-op values) 1-5 weeks after operation (Glasgow series).

Table B.10 Body weight changes up to 10 weeks after operation in pair-fed animals (Cape Town series).

Table B.11 Plasma amino acids 72 hours after control operation.

Table B.12 Plasma amino acids 72 hours after PCT.

Table B.13 Plasma amino acids 72 hours after PCS.

Table B.14 Plasma amino acids 10 weeks after control operation (*ad lib* fed rats).

Table B.15 Plasma amino acids 10 weeks after PCT (*ad lib* fed rats).

Table B.16 Plasma amino acids 10 weeks after PCS (*ad lib* fed rats).

Table B.17 Plasma amino acids 10 weeks after control operation in pair-fed rats.

Table B.18 Plasma amino acids 10 weeks after control operation in *ad lib* fed rats, used for hormone studies.

APPENDIX B, TABLE 1.

Body weight (as % of pre-operative weight) from 1 to 10 weeks after operation in ad lib fed rats (upper table and pair-fed rats (lower table). Values shown are means ± 1 SE_m.

AD LIB FED RATS

WEEKS	CONTROL	PCT	PCS
(n)	(15)	(10)	(10)
1	100.3 ± 1.6	94.1 ± 2.0	88.1 ± 3.0
2	108.5 ± 1.5	106.0 ± 2.2	86.8 ± 3.0
3	119.5 ± 2.0	118.2 ± 2.2	90.0 ± 2.6
4	126.1 ± 2.8	126.0 ± 3.4	89.3 ± 2.6
5	135.2 ± 2.2	133.8 ± 3.8	90.3 ± 3.5
6	146.1 ± 3.2	140.6 ± 4.2	92.4 ± 3.9
7	151.7 ± 3.4	144.9 ± 5.0	94.0 ± 4.6
8	158.5 ± 4.0	152.0 ± 6.2	96.2 ± 5.8
9	164.3 ± 4.2	156.3 ± 4.9	96.6 ± 5.3
10	170.2 ± 4.5	158.8 ± 4.5	98.3 ± 5.4

PAIR-FED RATS

WEEKS	CONTROL	PCT	PCS
(n)	(3)	(6)	(6)
1	96 ± 1.53	91 ± 2.4	91 ± 0.7
2	92 ± 6.4	91 ± 4.9	87 ± 3.2
3	96 ± 5.6	97 ± 3.7	91 ± 3.0
4	99 ± 7.6	100 ± 4.7	95 ± 2.9
5	106 ± 6.3	103 ± 4.4	97 ± 3.0
6	111 ± 8.6	105 ± 4.7	95 ± 2.3
7	103 ± 6.3	105 ± 4.4	99 ± 3.0
8	110 ± 9.0	108 ± 3.7	102 ± 3.1
9	106 ± 6.4	106 ± 3.6	104 ± 4.6
10	106 ± 6.9	105 ± 3.3	105 ± 4.1
11	104 ± 7.5	107 ± 2.8	106 ± 4.2
12	102 ± 6.8	108 ± 3.4	105 ± 4.1
13	107 ± 5.6	108 ± 3.0	108 ± 5.3
14	108 ± 5.9		

Liver wts (%/body wt)

Wet	2.55 ± 0.10	2.47 ± 0.10	1.83 ± 0.12
Dry	0.72 ± 0.12	0.74 ± 0.03	0.58 ± 0.02

APPENDIX B, TABLE 2.

Levels of ALT, LDH, CK and alkaline phosphatase up to 24 hours after control operation, PCT, PCS, laparotomy (LAP), anterior portal vein ligation (PVL), hepatic artery ligation (HAL), and partial hepatectomy (PH). Values shown for each of 3 rats per group.

		0 hrs			6 hrs			12 hrs			18 hrs			24 hrs		
ALT	C	47	56	52	134	200	158	94	102	107	65	66	70	86	1025	79
	PCT	54	68	86	167	158	264	102	120	197	89	73	132	78	58	135
	PCS	56	62	57	1490	460	818	2020	788	1388	1944	497	1007	1469	329	666
	LAP	74	92	71	58	81	59	44	82	36	48	82	38	48	75	38
	PVL	62	82	74	3209	1135	997	2402	931	995	2029	684	569	1422	435	373
	HAL	54	45	93	128	133	93	123	64	67	100	68	59	74	31	47
	PH	69	73	77	252	284	248	296	288	294	223	248	235	164	194	163
LDH	C	1242	----	746	2251	2862	2723	2617	899	2699	3593	2986	1703	1734	3772	944
	PCT	607	2564	838	1593	7776	1674	2164	4510	1634	1615	3956	286	3053	217	2753
	PCS	2260	1775	985	67288	10560	25338	64743	7061	14428	42290	4411	7329	17685	3441	2112
	LAP	2015	6808	4696	2421	2116	2913	952	1527	1295	452	635	210	2198	2751	4975
	PVL	2523	2571	1827	130590	35960	11575	22548	5777	5158	6945	8458	7329	3283	2706	5447
	HAL	652	1202	3695	1445	3250	2436	2641	2603	3195	3600	4980	3910	2163	8641	3757
	PH	2443	----	1986	1827	3147	3023	2491	2433	2259	2409	1675	1366	861	6376	187
CK	C	253	----	205	1049	1628	1297	1120	355	569	714	572	384	918	407	261
	PCT	284	695	475	1281	2354	831	901	995	532	525	1227	222	911	313	458
	PCS	875	825	368	5337	1584	1216	7433	711	680	2908	568	726	2765	1039	386
	LAP	671	1006	699	504	726	694	358	389	316	1071	932	235	409	522	1943
	PVL	548	594	296	866	1916	915	549	1235	530	93	1685	917	319	449	803
	HAL	164	351	1121	322	738	487	595	728	836	495	968	540	549	2040	751
	PH	420	----	534	426	793	701	393	660	469	657	618	835	364	1146	110
AP	C	282	442	319	302	405	351	308	378	257	226	378	300	299	396	218
	PCT	861	901	643	788	610	500	648	723	546	578	568	636	519	486	647
	PCS	651	809	191	528	789	----	731	743	370	749	716	350	868	634	332
	LAP	685	1113	642	468	1063	540	566	747	416	596	648	381	470	904	364
	PVL	605	946	923	597	725	726	639	603	829	535	488	546	706	471	532
	HAL	647	----	682	574	----	600	603	----	527	515	----	437	473	----	381
	PH	752	----	682	774	946	734	765	1206	832	1070	1442	990	1172	1296	983

APPENDIX B, TABLE 3

AST (u/l) 1-5 weeks after operation (Glasgow series)

	WEEKS					
	0	1	2	3	4	5
Control (20)	147	120	180	113	113	113
	177	163	159	110	116	276
	170	133	206	97	133	144
	177	244	244	146	180	216
	182	216	231	195	130	177
	225	169	230	170	250	209
	177	157	181	128	147	125
	198	188	143	106	230	340
	340	188	141	129	166	127
	192	243	173	204	279	110
	104	200	193	195	167	154
	105	157	168	161	156	139
	119	172	215	207	144	155
	158	185	216	140	148	178
	150	201	146	172	213	202
	118	123	207	166	112	146
	103	193	253	165	178	200
	347	204	180	108	187	102
	251	270	164	138	238	119
	287	233	171	116	214	112
PCT (13)	145	172	195	204	139	171
	162	182	159	173	192	132
	135	177	247	204	149	175
	104	195	264	167	122	130
	180	351	277	172	161	140
	186	197	192	187	295	89
	162	212	221	203	308	187
	170	219	194	184	166	173
	---	100	144	250	233	176
	145	131	231	183	198	146
	---	98	163	202	149	221
	130	110	111	143	138	206
	97	133	129	144	122	167
PCS (11)	138	140	173	166	102	273
	162	175	169	121	219	183
	149	196	345	246	269	960
	105	137	153	173	93	117
	119	121	174	241	171	130
	131	163	128	147	104	173
	227	151	306	181	208	151
	158	195	236	299	205	180
	135	138	248	224	137	125
	135	109	250	215	134	137
	143	349	328	282	238	203

APPENDIX B, TABLE 4

ALT (u/l) 1-5 weeks after operation (Glasgow series)

	WEEKS					
	0	1	2	3	4	5
Control (20)	53	41	45	39	57	51
	46	41	41	40	57	62
	51	34	41	41	61	61
	50	53	46	40	60	62
	45	44	45	37	75	59
	50	51	49	36	82	58
	37	39	42	33	46	37
	49	39	41	31	29	33
	--	39	52	45	43	38
	44	50	36	49	47	33
	57	45	43	53	45	47
	50	40	36	24	45	46
	42	26	29	37	61	41
	41	45	48	46	57	56
	56	57	61	49	58	50
	59	49	64	50	47	54
	40	46	44	27	57	56
	64	54	52	42	56	33
	56	72	42	36	48	36
	48	39	46	35	45	32
PTC (13)	37	36	28	44	37	45
	51	45	21	31	36	42
	44	40	45	50	44	53
	53	51	36	44	35	42
	59	39	48	46	43	41
	50	32	39	44	84	35
	37	36	44	35	46	38
	46	52	45	50	40	62
	--	39	31	43	40	43
	48	29	49	44	56	42
	--	25	36	48	41	47
	66	44	38	38	41	46
	40	44	37	39	36	39
PCS (11)	35	39	43	51	41	95
	36	29	35	33	35	40
	45	41	44	39	56	632
	35	40	33	38	63	49
	45	41	42	75	49	48
	48	50	43	49	40	59
	48	47	51	52	69	76
	39	64	69	69	42	41
	53	34	41	47	50	44
	48	36	49	46	48	43
	54	140	89	51	50	42

APPENDIX B, TABLE 5.

AP (u/l) 1-5 weeks after operation (Glasgow series)

	WEEKS					
	0	1	2	3	4	5
Control (20)	423	316	504	411	580	456
	372	324	458	415	530	487
	534	392	457	494	563	492
	299	267	360	417	498	427
	392	407	410	441	620	486
	295	384	364	392	415	411
	406	457	413	443	517	330
	579	541	536	346	469	376
	458	431	386	457	557	424
	355	443	403	461	608	397
	640	344	605	427	812	634
	443	288	431	269	557	403
	559	443	943	472	651	637
	434	229	542	409	449	472
	625	449	487	524	510	358
	575	441	440	474	513	541
	490	486	462	554	533	557
	469	358	572	483	511	350
	313	310	373	303	457	309
	500	500	460	508	542	381
PCT (13)	373	248	728	579	406	491
	427	310	799	1441	1113	891
	380	295	279	240	245	346
	523	563	1202	786	652	723
	671	914	524	541	525	447
	547	365	380	390	334	367
	409	282	259	266	375	367
	495	308	390	464	371	407
	---	403	456	511	499	554
	342	314	523	559	536	529
	---	699	595	663	591	723
	506	325	410	430	542	462
	317	326	287	405	367	376
PCS (11)	354	444	528	536	536	540
	440	1200	546	576	625	568
	417	481	655	559	468	498
	495	468	1158	629	802	977
	377	367	491	269	671	791
	342	388	623	851	781	697
	368	249	762	503	520	610
	568	506	468	733	993	428
	495	434	261	496	765	637
	496	414	1048	492	612	491
	490	405	368	1119	851	472

APPENDIX B, TABLE 6.

AST (u/l) 1-10 weeks after operation (UCT series)

	WEEKS										
	0	1	2	3	4	5	6	7	8	9	10
Control (14)	120	130	235	180	115	135	70	80	80	85	95
	85	110	115	145	220	124	110	90	160	95	125
	190	125	265	175	160	110	80	90	140	85	55
	205	100	135	115	100	120	100	70	90	70	205
	160	150	125	125	115	60	65	110	80	60	90
	180	100	125	150	170	130	105	110	90	105	140
	240	185	120	125	105	65	75	70	70	80	80
	110	180	185	150	135	105	65	85	70	95	90
	155	125	140	130	105	50	90	60	60	70	60
	120	90	90	115	140	120	110	105	135	120	110
	120	95	95	115	105	130	90	90	110	110	90
	75	85	95	145	105	140	100	105	155	85	45
	75	70	100	120	110	110	95	155	120	110	100
	100	---	150	120	120	111	100	110	120	140	80
PCT (10)	100	---	80	90	165	140	130	---	150	85	120
	---	80	110	125	120	---	---	---	---	---	---
	140	70	100	100	110	190	150	130	80	100	230
	---	60	160	160	195	140	130	105	120	95	90
	---	170	155	80	275	110	130	85	100	---	85
	55	195	80	60	120	90	95	65	75	110	105
	120	200	160	80	110	130	80	70	105	65	95
	150	70	55	95	90	50	70	105	100	115	110
	100	120	130	170	110	95	110	95	150	200	115
	90	200	115	90	75	80	80	120	80	140	---
PCS (10)	120	380	135	100	115	215	75	150	90	80	50
	100	210	155	---	115	120	55	110	105	90	70
	125	---	100	115	115	150	90	110	140	120	160
	---	240	150	155	215	140	95	110	95	105	85
	105	160	130	135	140	80	110	100	155	100	95
	95	205	165	130	110	80	110	90	90	90	70
	---	170	---	270	100	100	250	105	75	60	75
	80	120	---	185	90	160	70	90	135	110	85
	135	85	90	70	215	100	80	70	100	130	125
	105	95	80	80	140	125	---	---	---	---	---

APPENDIX B, TABLE 7.

Albumin levels 1-5 weeks after operation (Glasgow series). Values expressed as % pre-op value.

	WEEKS				
	1	2	3	4	5
SHAMS (n=19)	68.6	75.9	54.7	75.9	75.9
	77.4	103.0	77.4	80.1	72.2
	71.7	71.7	74.6	71.7	88.3
	100.0	116.9	97.5	103.2	97.5
	120.8	114.9	77.5	110.2	117.3
	100.0	100.0	93.8	106.4	113.3
	90.7	93.8	90.7	87.7	87.7
	64.5	90.5	87.5	117.0	109.9
	(299.6)	(320.7)	(376.3)	(400.4)	376.3)
	112.8	109.6	103.0	103.0	116.0
	77.7	82.8	82.8	94.0	85.5
	96.7	93.3	110.4	117.7	137.7
	74.9	45.1	100.0	117.3	117.3
	96.7	81.3	90.4	113.2	116.7
	88.4	94.2	91.2	100.0	72.1
	84.1	100.0	100.0	120.4	113.5
	75.1	60.2	62.5	83.0	94.1
	155.8	160.8	165.8	187.6	160.8
	100.0	109.3	88.2	103.2	100.0
	93.0	108.9	113.9	108.9	103.0
Mean	92.0	95.4	92.7	105.3	104.1
SD	± 21.5	± 24.6	± 23.1	± 24.9	± 22.4
SE	4.93	5.64	5.30	5.71	5.14
TRANSPOSITIONS (n=11)					
	103.9	124.2	108.1	71.5	88.8
	83.7	33.3	44.8	60.4	44.8
	76.1	82.7	82.7	92.8	100.0
	72.2	63.7	69.2	66.4	72.2
	77.1	74.0	74.0	77.1	88.3
	53.2	45.6	75.0	87.3	87.3
	43.0	56.6	74.4	69.0	77.0
	55.8	78.0	72.2	93.6	110.1
	80.1	90.0	110.6	80.1	83.3
	110.0	110.0	84.6	75.9	78.9
	118.7	92.5	107.3	107.3	118.7
Mean	79.4	77.3	82.1	80.1	86.3
SD	± 24.0	± 27.0	± 19.9	± 13.9	± 19.7
SE	7.24	8.14	6.00	4.19	5.94
SHUNTS (n=11)	90.5	87.6	84.6	84.6	96.9
	87.8	70.8	97.0	103.3	100.0
	73.0	81.8	81.8	42.7	70.2
	61.6	78.3	78.3	113.5	93.4
	44.1	56.5	75.3	84.4	64.2
	65.9	60.9	96.8	103.3	91.0
	71.5	28.9	63.2	68.6	65.8
	89.6	79.9	83.0	67.7	67.7
	70.8	50.5	67.7	110.6	107.1
	70.1	60.2	38.8	49.1	40.5
	90.6	36.3	52.2	64.8	73.0
Mean	74.1	62.9	74.4	81.1	79.1
SD	± 14.6	± 19.0	± 17.9	± 24.5	± 20.0
SE	4.40	5.73	5.40	7.39	6.03

APPENDIX B, TABLE 8.

Slow α_1 globulin 1-5 weeks after operation (Glasgow series). Values expressed as % pre-op value.

	WEEKS				
	1	2	3	4	5
SHAMS	104	101	124	147	149
	141	128	(59)	122	103
	122	144	127	122	141
	136	136	130	143	148
	125	113	—	93	101
	135	161	122	128	128
	107	111	100	113	154
	103	100	88	86	82
	—	—	—	—	—
	127	109	109	136	108
	—	121	134	121	129
	139	132	116	96	104
	124	120	118	97	116
	107	133	107	108	100
	118	105	105	114	141
	96	105	132	113	129
	100	118	93	101	97
	—	—	—	—	—
	97	107	100	93	96
	116	100	105	105	100
	(n=17)	(n=18)	(n=16)	(n=18)	(n=18)
Mean	117	119	113	113	118
SD	15.2	17.0	14.3	17.7	22.0
SE	3.7	4.0	3.6	4.2	5.2
TRANSPOSITIONS (n=11)					
	113	105	113	118	104
	119	111	140	125	110
	118	123	104	91	88
	116	106	104	100	100
	130	130	122	125	100
	104	84	115	140	140
	100	111	119	111	115
	109	115	122	113	106
	117	100	122	122	127
	109	92	109	130	122
	159	102	105	119	119
Mean	118	107	116	118	112
SD	15.6	13.1	10.8	13.7	14.6
SE	4.7	3.9	3.2	4.1	4.4
SHUNTS (n=10)	142	124	99	99	79
	121	115	111	93	96
	112	120	115	112	130
	119	103	94	84	73
	89	100	104	82	82
	116	112	133	112	95
	113	106	104	97	97
	111	103	98	91	87
	104	113	100	113	81
	125	122	110	111	106
	—	—	—	—	—
Mean	115	112	107	99	93
SD	13.8	8.6	11.3	12.0	16.5
SE	4.4	2.7	3.6	3.8	5.2

APPENDIX B, TABLE 9.

IgG levels 1-5 weeks after operation (Glasgow series).
Values expressed as % pre-op value.

	WEEKS				
	1	2	3	4	5
SHAMS (n=19)	70.0	104.4	108.9	118.4	219.2
	63.1	96.3	66.3	94.3	72.5
	46.5	64.2	66.9	64.2	61.6
	56.8	87.1	73.2	76.3	83.6
	100.0	100.0	100.0	114.8	114.8
	89.5	105.5	116.6	153.3	181.1
	103.2	82.7	95.3	132.8	127.6
	74.4	104.9	100.0	104.9	124.1
	100.0	158.3	108.7	138.0	219.9
	76.8	80.6	108.4	121.6	112.5
	100.0	127.4	135.3	151.6	158.1
	102.2	122.1	171.5	145.6	160.9
	52.8	104.4	132.8	153.3	175.0
	103.8	124.7	119.6	103.8	120.5
	85.2	100.0	116.1	137.1	132.8
	80.1	131.5	141.0	166.0	166.0
	185.6	194.4	194.4	176.7	185.6
	91.4	100.0	95.4	104.4	108.7
	82.3	123.4	118.4	133.2	123.4
Mean	87.56	111.15	114.7	125.81	139.36
SD	± 29.76	± 29.41	± 32.67	± 29.78	± 45.00
SE	6.83	6.75	7.50	6.83	10.32
TRANSPOSITIONS (n=11)					
	133.8	113.4	246.4	312.7	333.0
	82.8	61.7	208.2	257.0	265.6
	190.2	254.4	276.4	288.3	300.3
	110.5	127.3	139.5	157.9	204.7
	127.3	157.9	190.5	197.9	197.9
	83.2	150.2	164.5	171.4	258.2
	80.0	164.2	204.7	184.2	177.4
	100.0	186.6	203.2	194.4	211.2
	77.2	100.0	104.0	112.4	112.4
	92.0	108.6	122.1	140.9	140.9
	163.2	178.9	211.2	273.9	264.7
Mean	112.75	145.74	188.24	208.27	224.21
SD	± 37.28	± 51.95	± 51.95	± 65.19	± 67.09
SE	± 11.24	± 15.67	± 15.66	± 19.65	± 20.23
SHUNTS (n=11)	100.0	170.6	177.5	184.4	170.6
	94.8	116.4	151.6	177.5	105.4
	78.8	105.8	111.7	78.8	176.3
	48.4	84.8	133.7	122.0	121.0
	76.1	202.8	285.8	186.6	194.4
	93.7	177.6	244.6	226.5	201.3
	143.9	157.7	241.4	258.2	250.8
	134.2	205.9	302.8	280.4	320.7
	74.5	153.5	166.7	187.3	201.2
	94.4	186.2	164.5	224.7	241.4
	88.2	157.7	193.6	186.2	186.2
Mean	93.36	156.27	197.62	192.05	197.21
SD	± 26.8	± 39.29	± 62.47	± 57.03	± 59.68
SE	8.08	11.85	18.83	17.20	17.99

Appendix B, Table 10: Body weights (G) after PCS, PCT or control operation in Cape Town pair feeding study.

WEEK	PCS1	PCS2	PCS3	PCS4	PCS5	PCS6
0	319	223	302	311	311	316
1	265	289	257	289	295	286
2	263	247	265	268	308	293
3	284	278	287	267	326	284
4	306	300	280	279	338	306
5	306	305	282	285	346	302
6	293	301	279	292	330	315
7	287	308	289	315	344	334
8	280	335	297	337	340	331
9	270	341	287	342	344	365
10	272	340	304	345	343	362
11	282	328	298	347	357	365
12	283	335	293	347	345	364
13	285	335	295	346	373	389
14	277	334	289	368	370	377
15	---	341	---	---	---	---

WEEK	PCT1	PCT2	PCT3	PCT4	PCT5	PCT6
1	288	314	320	244	352	308
2	258	284	285	252	318	264
3	244	264	271	277	327	289
4	251	298	292	276	358	284
5	267	318	274	292	355	321
6	280	324	288	300	360	322
7	289	335	292	304	376	332
8	289	336	306	297	325	324
9	299	371	319	294	361	318
10	286	365	315	285	349	331
11	286	359	315	280	334	319
12	295	351	317	284	349	339
13	293	354	331	299	364	319
14	293	338	333	288	355	362
15	305	357	344	---	---	357

WEEK	SHAM1	SHAM3
1	319	330
2	301	324
3	259	338
4	272	336
5	285	322
6	351	322
7	389	331
8	388	315
9	369	301
10	350	305
12	351	303
13	344	293
14	335	294
15	335	328
16	351	320

APPENDIX B, TABLE 11.

Plasma amino acids (μmol/ml) 72 hours after control operation.

				B			A			
Control	VAL	ILEU	LEU	(V+I+L)	TYR	PHE	(T+P)	B/A	GLY	ALA
	104	58	88	250	70	44	144	1.74	326	---
	162	66	132	360	64	54	118	3.05	296	336
	134	48	114	296	66	56	122	2.43	336	378
	134	64	126	324	66	58	124	2.61	276	482
	86	46	106	238	74	52	126	1.89	336	482
	310	72	174	556	80	56	136	4.09	510	626
	170	62	182	414	74	80	154	2.69	338	564
	128	26	128	282	50	56	106	2.66	290	390
	122	26	86	234	48	166	214	1.09	336	368
	106	57	85	248	69	74	143	1.73	659	429
	110	40	102	252	52	68	140	1.80	422	438
Mean	142	51	120		64	69		2.35	375	449
SEm	18	5	10		3	10		0.25	34	29

APPENDIX B, TABLE 12.

Plasma amino acids (μmol/ml) 72 hours after PCT.

				B			A			
PCT	VAL	ILEU	LEU	(V+I+L)	TYR	PHE	(T+P)	B/A	GLY	ALA
	78	38	72	188	74	64	138	1.36	414	486
	81	37	64	182	63	63	126	1.44	420	420
	102	52	113	267	63	73	136	1.96	375	398
	92	32	64	188	52	60	112	1.68	380	318
	168	64	128	360	100	92	192	1.88	508	738
	136	72	132	340	104	92	196	1.73	398	722
	86	30	70	186	60	64	124	1.50	396	432
	104	44	92	240	63	48	111	2.16	334	366
	72	32	64	168	59	68	127	1.32	385	518
	122	56	114	292	74	69	143	2.04	325	516
	104	42	79	225	66	68	134	1.68	330	396
Mean	105	45	90		71	69		1.70	388	483
SEm	8	4	8	5	5	4		0.08	16	41

APPENDIX B, TABLE 13.

Plasma amino acids (μmol/ml) 72 hours after PCS.

				B			A			
PCS	VAL	ILEU	LEU	(V+I+L)	TYR	PHE	(T+P)	B/A	GLY	ALA
	60	38	44	142	104	82	186	0.76	344	532
	66	42	52	160	70	78	148	1.08	266	300
	100	48	100	248	130	104	234	1.06	346	440
	113	58	100	271	102	87	189	1.43	305	543
	84	48	24	156	112	98	210	0.74	362	422
	116	58	98	272	112	118	230	1.18	424	442
	104	40	80	224	84	92	176	1.27	352	518
	108	48	100	256	118	148	266	0.96	310	436
	68	26	64	158	88	78	166	0.95	322	420
	92	30	84	206	168	96	264	0.78	298	528
	80	40	62	182	112	98	210	0.87	408	424
	54	36	68	158	86	90	176	0.90	302	414
Mean	87	43	73		107	97		1.00	337	452
SEm	6	3	7		7	6		0.06	13	20

APPENDIX B, TABLE 14.

Plasma amino acids (μmol/ml) 10 weeks after control operation in ad lib fed rats.

				A			B			
Control	VAL	ILEU	LEU	(V+I+L)	TYR	PHE	(T+P)	B/A	GLY	ALA
	228	98	188	514	84	66	150	3.43	288	440
	235	109	228	572	84	91	175	3.27	416	576
	65	34	66	165	39	39	78	2.12	136	162
	175	77	149	401	63	67	130	3.08	307	405
	199	101	182	482	77	69	146	3.30	224	475
	141	63	120	324	67	57	124	2.61	234	282
	206	80	182	468	71	73	144	3.25	197	417
	194	78	149	421	58	59	117	3.60	176	310
	164	64	124	352	43	45	88	4.00	198	274
	192	86	182	460	67	83	150	3.07	232	432
	75	34	68	177	29	29	58	3.05	105	145
	125	82	125	332	51	49	100	3.22	170	259
	—	80	145	—	57	53	110	—	148	305
	128	65	121	314	57	61	118	2.66	187	265
Mean	164	75	145		61	60		3.14	216	339
SEm	15	6	12		4	5		0.13	21	33

APPENDIX B, TABLE 15.

Plasma amino acids (μmol/ml) 10 weeks after PCT in ad lib fed rats.

				B			A			
PCT	VAL	ILEU	LEU	(V+I+L)	TYR	PHE	(T+P)	B/A	GLY	ALA
	150	68	134	352	80	88	168	2.10	198	438
	244	96	200	540	90	84	174	3.10	376	738
	164	94	212	470	90	110	200	2.35	328	490
	107	51	88	246	67	64	131	1.88	234	339
	180	69	171	420	45	82	127	3.31	248	374
	112	40	107	259	59	68	127	2.04	314	366
	129	50	126	305	61	71	132	2.31	295	330
	48	65	136	249	80	81	161	1.55	298	409
	77	31	63	171	40	43	83	2.06	126	173
Mean	135	63	137		68	77		2.30	269	406
SEm	19	7	17		6	6		0.19	25	51

APPENDIX B, TABLE 16.

Plasma amino-acids (μmol/ml) 10 weeks after PCS in ad lib fed rats

				B			A			
PCS	VAL	ILEU	LEU	(V+I+L)	TYR	PHE	(T+P)	B/A	GLY	ALA
	75	28	61	164	97	82	179	0.92	235	257
	106	37	69	212	101	90	191	1.11	229	274
	94	40	72	206	92	64	156	1.32	263	281
	85	34	63	182	81	75	156	1.17	271	281
	81	34	73	188	77	91	168	1.12	427	284
	104	36	88	228	105	107	212	1.08	383	426
	133	57	132	322	85	108	193	1.67	329	416
	106	43	102	251	118	107	225	1.12	409	443
	93	43	90	227	87	91	178	1.28	217	282
Mean	97	39	83		94	91		1.20	307	327
SEm	6	2	8		4	5		0.07	27	26

APPENDIX B, TABLE 17.

Plasma amino acid levels (μmol/ml) 10 weeks after operation in pair-fed rats.

				B			A			
Control	VAL	ILEU	LEU	(V+I+L)	TYR	PHE	(T+P)	B/A	GLY	ALA
	84	44	70	198	24	32	56	3.54	220	196
	76	66	112	254	38	58	96	2.65	306	204
	92	52	108	252	44	62	106	2.38	204	298
Mean	84	54	97		35	51		2.86	243	233
SEm	4	6	13		6	9		0.35	32	33
PCT	—	42	76	—	44	60	104	—	200	206
	76	46	90	212	46	38	84	2.52	276	264
	80	44	78	202	58	58	116	1.74	280	260
	40	40	56	136	38	48	86	1.58	208	152
	52	40	60	152	24	50	74	2.05	284	216
Mean	62	42	72		42	58		1.97	250	220
SEm	9	1	6		6	4		0.20	94	20
PCS	62	38	76	176	70	62	132	1.33	196	224
	78	40	86	204	74	70	144	1.42	176	222
	60	46	82	188	76	68	144	1.31	244	228
	120	56	108	284	90	102	192	1.48	364	446
	58	42	66	166	44	44	88	1.89	292	286
	56	38	78	172	88	94	182	0.95	200	276
Mean	72	43	83		74	73		1.40	245	280
SEm	10	3	6		7	9		0.57	29	35

APPENDIX B, TABLE 18.

Plasma amino acids (μmol/ml) 10 weeks after operation in ad lib fed rats. This second series of animals was used for the hormone studies. Results are not significantly different from Chapter V, Table 5.3.

Control	VAL	ILEU	LEU	B (V+I+L)	TYR	PHE	A (T+P)	B/A	GLY	ALA
	192	66	134	392	104	86	190	2.06	266	424
	214	80	156	450	76	72	148	3.04	228	410
	274	120	268	662	110	104	214	3.09	294	500
	240	118	204	562	80	90	170	3.30	260	444
Mean	230	96	191		93	88		2.87	262	445
SEm	18	14	30		8	6		0.28	14	20
PCT	146	60	116	322	88	72	160	2.01	—	—
	142	62	102	306	100	98	198	1.55	284	402
	164	74	144	382	70	84	154	2.48	206	436
	142	64	116	322	74	86	160	2.01	278	396
	140	54	92	286	76	80	156	1.83	190	408
Mean	147	63	114	8	82	84		1.98	240	411
SEm	4	3	9		5	4		0.15	24	9
PCS	112	60	90	262	126	126	252	1.04	336	380
	136	60	60	256	104	106	210	1.22	280	512
	138	48	106	292	102	98	200	1.46	240	396
	134	36	104	274	138	94	232	1.18	276	426
	96	44	98	238	103	102	205	1.16	240	334
Mean	123	50	92		115	105		1.21	274	410
	8	5	8		7	5		0.07	18	30

APPENDIX B, TABLE 19.

Plasma and brain NH_3 levels 10 weeks after operation in ad lib fed rats.

		PLASMA (μmol/ml)	BRAIN (μmol/g)		
	Rat No.		Cerebrum	Brain stem	Cerebellum
Control	21	7.8	0.33	1.14	1.08
	22	11.0	0.78	0.92	0.69
	23	10.1	0.55	——	1.19
	25	——	0.61	1.24	1.07
	26	——	0.59	1.55	1.10
	27	2.8	0.48	0.99	2.11
	Mean	7.9	0.56	1.16	1.21
	SEm	± 2.0	± 0.06	± 0.11	± 0.19
PCT	22	——	0.77	1.64	——
	23	16.1	0.28	0.68	——
	26	15.5	0.63	0.63	0.36
	27	21.9	——	1.04	1.51
	28	30.3	0.24	0.77	1.35
	29	18.0	0.51	0.52	0.88
	30	9.9	0.34	1.84	2.24
	Mean	18.6	0.46	1.02	1.27
	SEm	± 2.8	± 0.09	± 0.20	± 0.32
PCS	21	——	0.54	1.72	1.92
	22	21.5	0.46	1.75	2.51
	24	22.5	0.58	0.86	——
	25	12.9	0.58	2.50	2.48
	26	5.0	——	1.30	2.37
	27	6.2	0.77	3.20	2.02
	28	23.2	0.29	1.36	1.32
	29	18.4	0.46	2.97	1.51
	Mean	15.7	0.53	1.96	2.02
	SEm	± 2.9	± 0.06	± 0.30	± 0.18

APPENDIX B, TABLE 20.

Plasma and brain NH_3 levels 10 weeks after operation in pair-fed rats.

		PLASMA (μmol/ml)	BRAIN (μmol/g)		
	Rat No.		Cerebrum	Brain stem	Cerebellum
Control	1	5.4	0.32	0.63	1.54
	2	6.7	1.07	6.41	8.06
	3	4.4	——	——	——
	Mean	5.5	0.70	3.52	4.80
	SEm	± 0.7	——	——	——
PCT	1	9.5	0.77	1.65	1.45
	2	9.5	0.82	1.49	1.39
	3	9.9	0.69	1.56	2.10
	4	9.0	2.49	3.09	10.92
	5	7.7	0.64	7.77	2.52
	6	8.4	——	——	——
	Mean	9.0	1.08	3.11	3.68
	SEm	± 0.3	± 0.35	± 1.20	± 1.82
PCS	1	11.2	0.56	2.24	1.45
	2	10.9	1.35	1.69	1.10
	3	10.9	0.34	1.54	1.83
	4	12.7	0.78	2.94	6.75
	5	11.1	1.41	4.05	6.13
	6	11.4	——	——	——
	Mean	11.4	0.89	2.49	3.45
	SEm	± 0.3	± 0.21	± 0.46	± 1.23

APPENDIX B, TABLE 21.

Plasma and brain NH_3 levels 72 hours after operation.

		PLASMA (μmol/ml)	BRAIN (μmol/g)		
	Rat No.		Cerebrum	Brain stem	Cerebellum
Control	31	——	0.74	0.98	0.95
	33	16.8	0.54	0.65	0.82
	Mean	16.8	0.64	0.82	0.89
	SEm	——	——	——	——
PCT	31	6.8	0.10	0.60	0.51
	32	19.5	1.54	1.75	1.37
	33	10.6	1.90	1.85	0.41
	Mean	12.3	1.18	1.40	0.76
	SEm	± 3.8	± 0.55	± 0.69	± 0.30
PCS	31	16.2	0.91	1.58	1.21
	32	12.8	0.81	0.57	——
	33	19.5	0.68	1.55	1.88
	34	11.6	0.30	1.41	0.83
	35	20.9	0.63	1.36	1.30
	36	13.7	——	——	——
	37	14.5	——	——	——
	38	18.1	——	——	——
	Mean	15.9	0.67	1.29	1.31
	SEm	± 1.2	± 0.10	± 0.19	± 0.22

APPENDIX B, TABLE 22.

Glutamate and glutamine (μmol/g wet weight) in the brain 10 weeks after operation in ad lib fed rats.

		GLUTAMATE			GLUTAMINE		
	Rat No.	Cerebrum	Brain Stem	Cerebellum	Cerebrum	Brain Stem	Cerebellum
Control	21	8.42	9.72	10.13	11.17	10.07	14.47
	22	5.61	5.83	14.57	7.62	5.30	15.32
	23	9.03	----	9.13	10.86	-----	10.44
	25	6.42	4.86	7.87	8.11	5.74	12.81
	26	6.88	7.75	9.10	5.36	6.50	11.50
	27	7.88	5.06	6.34	6.55	5.63	10.04
	Mean	7.37	6.64	9.52	8.28	6.65	12.43
	SEm	± 0.53	± 0.92	± 1.12	± 0.95	± 0.88	± 0.88
PCT	22	9.88	6.31	-----	20.53	16.76	-----
	23	9.14	5.01	9.37	13.01	10.24	17.56
	26	8.56	9.68	-----	9.36	0.88	18.18
	27	----	6.04	6.91	-----	3.83	-----
	28	6.07	4.40	10.58	11.56	8.60	23.72
	29	7.69	6.53	8.98	2.97	13.22	5.61
	30	2.29	4.04	3.78	12.58	8.59	13.40
	Mean	7.27	6.00	7.92	11.67	8.87	15.69
	SEm	± 1.13	± 0.71	± 1.19	± 2.32	± 2.03	± 3.01
PCS	21	6.60	7.86	10.09	20.24	29.81	32.18
	22	3.86	2.86	11.12	6.37	7.98	31.93
	24	7.12	9.23	7.72	9.16	13.00	11.37
	25	1.59	1.88	0.76	3.55	5.44	6.37
	26	----	3.25	3.49	-----	3.46	6.25
	27	5.44	5.79	6.72	4.35	5.18	9.39
	28	7.75	6.10	7.71	4.80	6.70	6.77
	29	7.37	7.43	7.12	6.42	13.30	5.29
	Mean	5.68	5.55	6.84	7.84	10.61	13.69
	SEm	± 0.85	± 0.93	± 1.18	± 2.18	± 3.00	± 4.07

APPENDIX B, TABLE 23.

Tryptophan (μmol/g) in brain 10 weeks after operation in ad lib fed rats.

		TRYPTOPHAN (μmol/g)		
	Rat No.	Cerebrum	Brain Stem	Cerebellum
Control	21	59.1	331.1	150.2
	22	37.8	182.4	99.2
	23	120.9	-----	238.7
	25	17.7	65.3	51.9
	26	15.6	69.6	40.1
	27	44.5	48.2	93.0
	Mean	32.6	139.3	112.2
	SEm	± 7.1	± 53.5	± 29.9
PCT	22	53.2	118.6	-----
	23	63.7	167.5	162.2
	26	43.3	128.5	-----
	27	----	89.9	106.0
	28	61.6	112.6	231.5
	29	54.2	104.1	184.8
	30	73.3	276.2	135.3
	Mean	58.2	142.5	164.0
	SEm	± 4.2	± 24.1	± 21.4
PCS	21	21.1	57.9	89.0
	22	29.5	85.2	187.3
	24	56.0	144.5	154.6
	25	36.6	126.5	-----
	26	----	301.6	204.8
	27	88.6	150.9	91.6
	28	44.0	93.2	96.8
	29	42.5	141.7	97.9
	Mean	45.5	137.7	131.7
	SEm	± 8.3	± 26.2	± 18.7

APPENDIX B, TABLE 24.

Brain amino acid levels in pair-fed rats 10 weeks after operation. Values are given as μmol/g wet weight.

Rat	Region	VAL	ILEU	LEU	PHE	TYR	MET	GLY	ALA
SH 1	Cerebrum	-----	.0277	.0555	.0697	.0484	.0206	-----	-----
SH 1	Bel	.2608	.0405	.0648	.1085	.0988	-----	-----	-----
SH 1	Brainstem	.0696	.0228	.0664	.0544	.0500	-----	-----	-----
SH 2	Cerebrum	.6186	.4068	.9141	.9141	.7078	.3622	.3600	.3032
SH 2	Bel	3.245	1.168	3.118	2.986	2.336	.6500	5.845	7.013
SH 2	Brainstem	-----	1.210	2.063	1.706	1.637	.8532	-----	-----

APPENDIX B, TABLE 25.

Brain amino acid levels in pair-fed rats 10 weeks after operation. Values are given as μmol/g wet weight.

Rat	Region	VAL	ILEU	LEU	PHE	TYR	MET	GLY	ALA
PCT 1	Cerebrum	.1688	.0272	.0719	.1125	.0887	.0307	-----	-----
PCT 1	Bel	-----	.0154	.0280	.0793	.0629	-----	-----	-----
PCT 1	Brainstem	.1354	-----	.0815	.0913	.0619	-----	-----	-----
PCT 2	Cerebrum	-----	.0484	.0609	.1076	.0947	.0317	-----	-----
PCT 2	Bel	-----	-----	.0384	.0855	.1091	-----	-----	-----
PCT 2	Brainstem	-----	-----	.0890	.1780	.1342	-----	-----	-----
PCT 3	Cerebrum	-----	.0315	.0894	.0789	.0789	-----	-----	-----
PCT 3	Bel	-----	-----	-----	-----	-----	-----	-----	-----
PCT 3	Brainstem	.1019	.0200	.0717	.0775	.0976	.0258	-----	-----
PCT 4	Cerebrum	-----	1.516	3.445	1.764	1.474	.8546	-----	-----
PCT 4	Bel	.8939	.3245	.8142	1.218	1.098	.2846	2.767	3.296
PCT 4	Brainstem	2.436	1.456	3.129	2.246	1.338	.5502	8.720	4.873
PCT 5	Cerebrum	1.654	.8687	1.991	1.196	.9425	.5900	4.146	4.064
PCT 5	Bel	2.266	1.359	2.968	2.350	1.278	.9071	7.173	5.194
PCT 5	Brainstem	2.543	1.164	2.754	2.436	1.271	.6359	5.934	3.923

APPENDIX B, TABLE 26.

Brain amino acid levels in pair-fed rats 10 weeks after operation. Values are given as μmol/g wet weight.

Rat	Region	VAL	ILEU	LEU	PHE	TYR	MET	GLY	ALA
PCS 1	Cerebrum	.0341	.0243	.0544	.1049	.1293	.0341	-----	-----
PCS 1	Bel	-----	-----	.0183	.0658	.0871	-----	-----	-----
PCS 1	Brainstem	-----	.1276	-----	.1276	.1811	-----	-----	-----
PCS 2	Cerebrum	-----	-----	-----	.1610	.1610	-----	-----	-----
PCS 2	Bel	-----	.0603	.0794	.2288	.2383	-----	.8645	.7453
PCS 2	Brainstem	-----	-----	.1093	.1863	.1565	-----	-----	-----
PCS 3	Cerebrum	.0468	.0468	.0975	.1328	.1719	.0391	-----	-----
PCS 3	Bel	.1287	.0253	.0580	.1885	.2465	-----	-----	-----
PCS 3	Brainstem	4.911	-----	2.232	6.228	7.272	-----	-----	-----
PCS 4	Cerebrum	1.182	.7763	.1833	.9243	.7096	.3106	2.654	3.216
PCS 4	Bel	.2908	-----	-----	-----	-----	-----	1.200	1.532
PCS 4	Brainstem	1.638	1.054	2.184	1.601	.8989	.4307	5.072	3.745
PCS 5	Cerebrum	1.797	1.650	3.228	1.760	1.394	.6603	6.200	5.759
PCS 5	Bel	-----	.8898	1.906	.9321	.8898	.5084	-----	-----
PCS 5	Brainstem	2.260	1.443	3.075	2.071	1.254	.3141	4.819	5.146

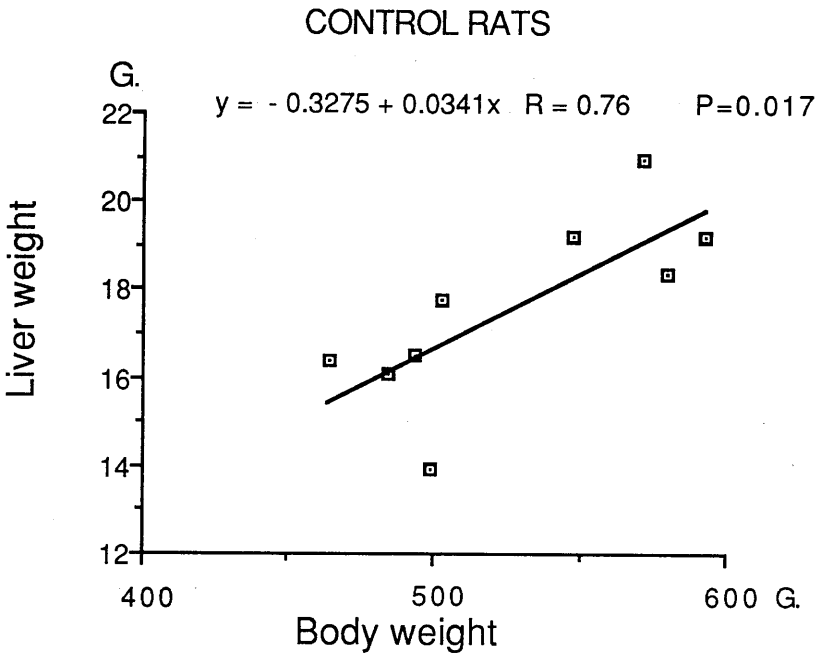


Fig. B.1 Liver weight vs. body weight at sacrifice 10 weeks after operation in Control rats. Statistics shown are regression equation and coefficient of correlation, with P value.

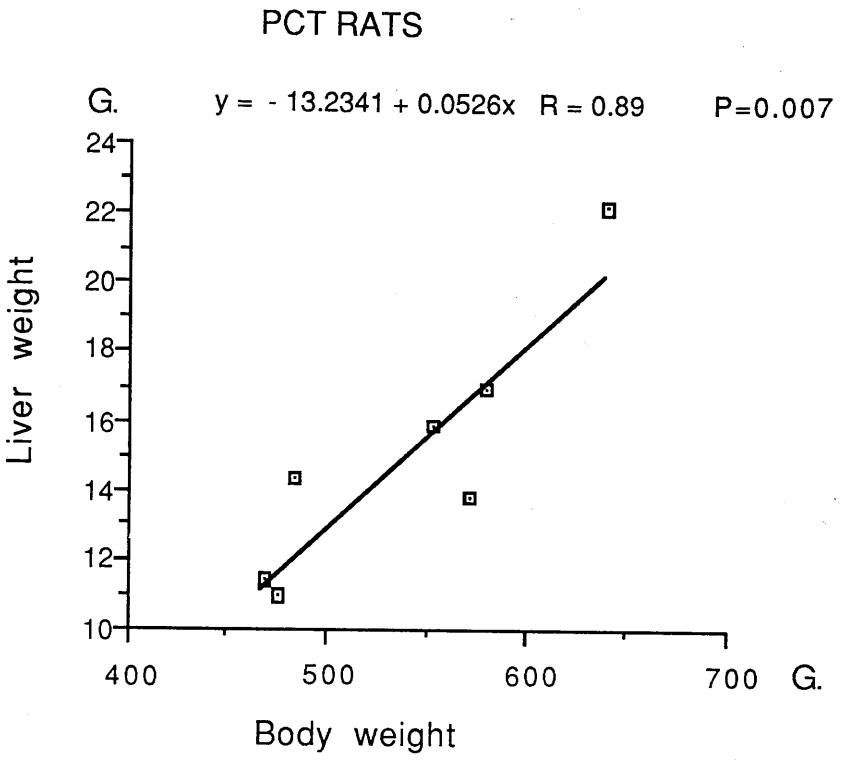


Fig. B.2 Liver weight vs. body weight at sacrifice 10 weeks after operation in PCT rats. Statistics shown are regression equation and coefficient of correlation, with P value.

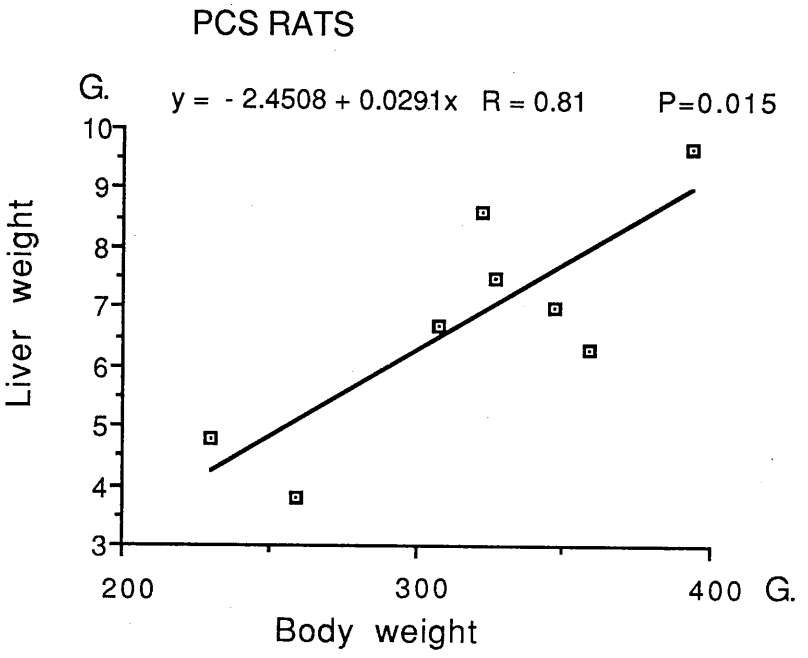


Fig. B.3 Liver weight vs. body weight at sacrifice 10 weeks after operation in PCS rats. Statistics shown are regression equation and coefficient of correlation, with P value.

APPENDIX C.

Reprints of papers bound after Reference section.

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Portacaval transposition in the rat: a new technique and its effects on liver and body weight

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SUMMARY

Portacaval transposition by direct end-to-end anastomoses has been established in rats, with negligible mortality and with survivors up to 150 days. The operation was associated with an early fall in body weight, followed by a recovery period the rapidity of which was related to the preoperative weight (and thus age) of the animal. At sacrifice after 35 days the relative liver weight of the animals with portacaval transposition was significantly reduced ($P<0{\cdot}01$), though it remained significantly higher ($P<0{\cdot}001$) than that in a group of animals with a portacaval shunt. The only histological change was minimal congestion of the sinusoids in the animals with portacaval transposition.

PORTACAVAL transposition offers considerable theoretical advantage over conventional portal-systemic shunting procedures in the treatment of portal hypertension, since liver perfusion is maintained. Technical problems have, however, limited its application, and its clinical use has been reported in only a few instances. As an experimental preparation portacaval transposition provides a useful tool for the investigation of hepatic metabolism, and specifically of the mechanism of hepatic encephalopathy. Child et al. first described portacaval transposition in the dog in 1953, and their preparation has since been widely used. Lecompte et al. (1970) reported a method for portacaval transposition in the rat in which a left nephrectomy was necessary. We have developed a technique for portacaval transposition in the rat using direct end-to-end anastomoses, and have studied the effects of the procedure on body weight and on liver weight. Preliminary studies of liver histology have also been carried out.

Materials and methods

Male Sprague–Dawley rats weighing between 270 and 405 g were anaesthetized with ether and intraperitoneal Veterinary Nembutal (0·07 ml/100 g body weight). All the procedures were clean but not sterile and antibiotics were not administered. Postoperatively the animals were caged in groups of 4, under controlled conditions of temperature and humidity, and fed on rat cube diet 41B.

Surgical technique

The abdomen is opened through a midline incision. The median and right lateral lobes of the liver are mobilized and delivered upwards on to the anterior abdominal wall to provide access to the vena cava and the portal vein. The intestines are displaced to the left of the animal, exposing the portal vein and vena cava.

Vascular dissection and anastomoses are performed using × 10 magnification with a Zeiss operating microscope, and 7–0 silk mounted on an 8-mm atraumatic needle (Ethicon W6594). Tearing of the vessels, with consequent leakage, is avoided by constant moistening of the operative field with sterile water. The vena cava is mobilized and meticulously cleaned from the junction of the right renal vein to the inferoposterior margin of the liver. The right gastric vein—the most proximal tributary of the portal vein—is ligated using 6–0 silk, and the portal vein is mobilized and meticulously cleaned along its length. The vena cava is then divided between clamps, one placed proximal to the right renal vein and the other flush with the lower border of the liver. The portal vein is also divided between clamps applied so as to give the maximum possible length of vein free for manipulation. Two stay sutures are placed so as to attach the distal portal vein to the proximal vena cava at opposite points on their circumference. The ends are left free and gentle traction is applied throughout the procedure. First the posterior and then the anterior walls are anastomosed using a continuous over-and-over suture (*Fig.* 1). Pressure is applied to the anastomotic line with a cotton wool pledget while the clamps are removed, and the vessels are finally pumped gently with a cotton wool pledget to free any clots present. The second anastomosis, of the distal vena cava to the proximal portal vein, is carried out in a precisely similar manner (*Fig.* 2). Some manipulation of the liver and clamped vessels may be necessary to achieve approximation of the vessels without tension (*Fig.* 3). The total duration of occlusion of the portal vein was not allowed to exceed 15 minutes. The duration of vena caval occlusion in practice did not exceed 35 minutes, though this time is probably not critical. The abdomen is closed with catgut for muscle and metal clips for the skin.

Experimental design

A preliminary study was first performed. Portacaval transposition was carried out in 9 animals, with 2 intra-operative deaths. Four animals were sacrificed

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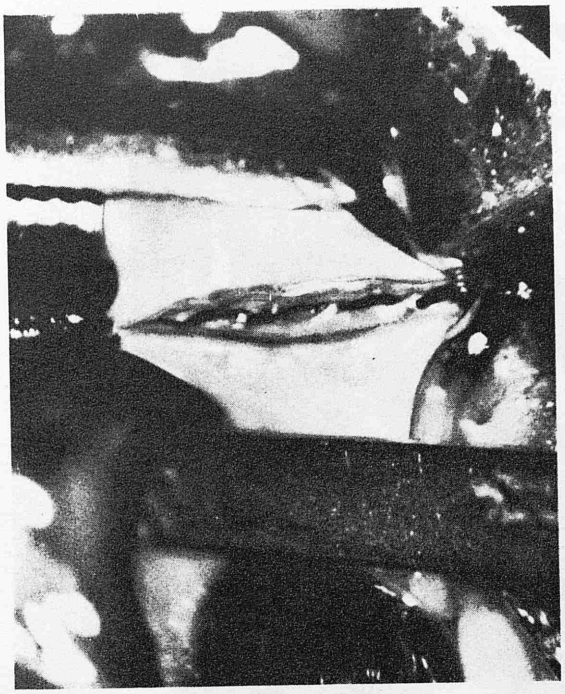

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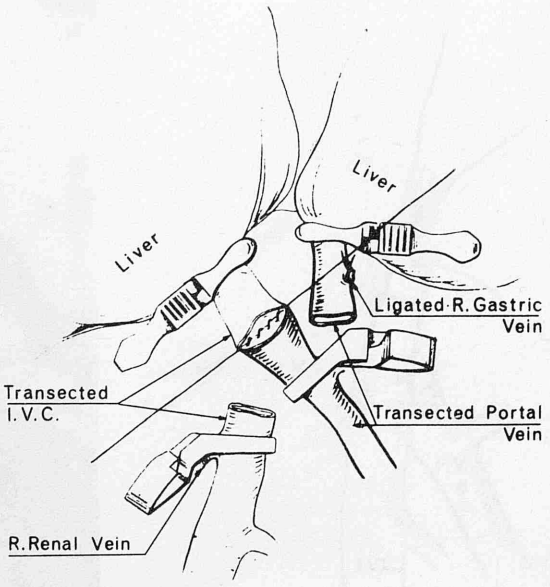

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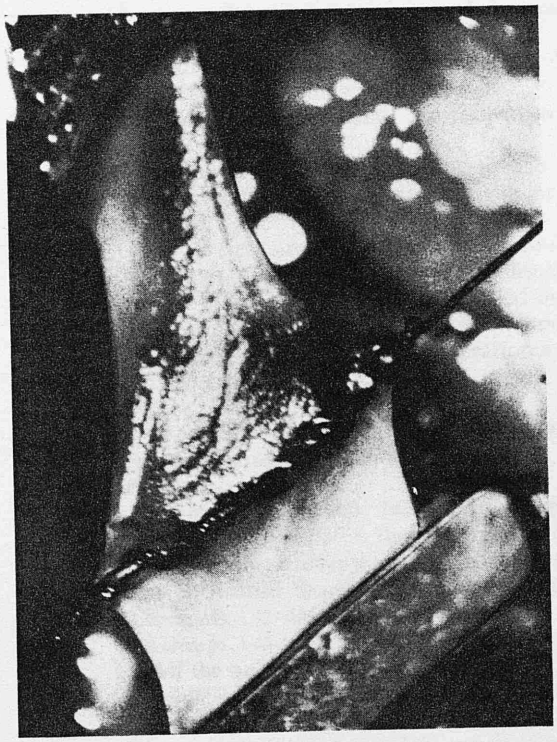
Fig. 1. Suturing of the distal portal vein to the proximal inferior vena cava (posterior layer). Note the importance of stay sutures.



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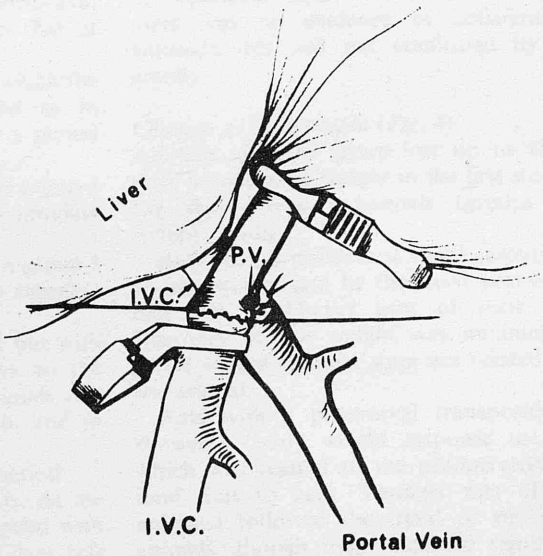

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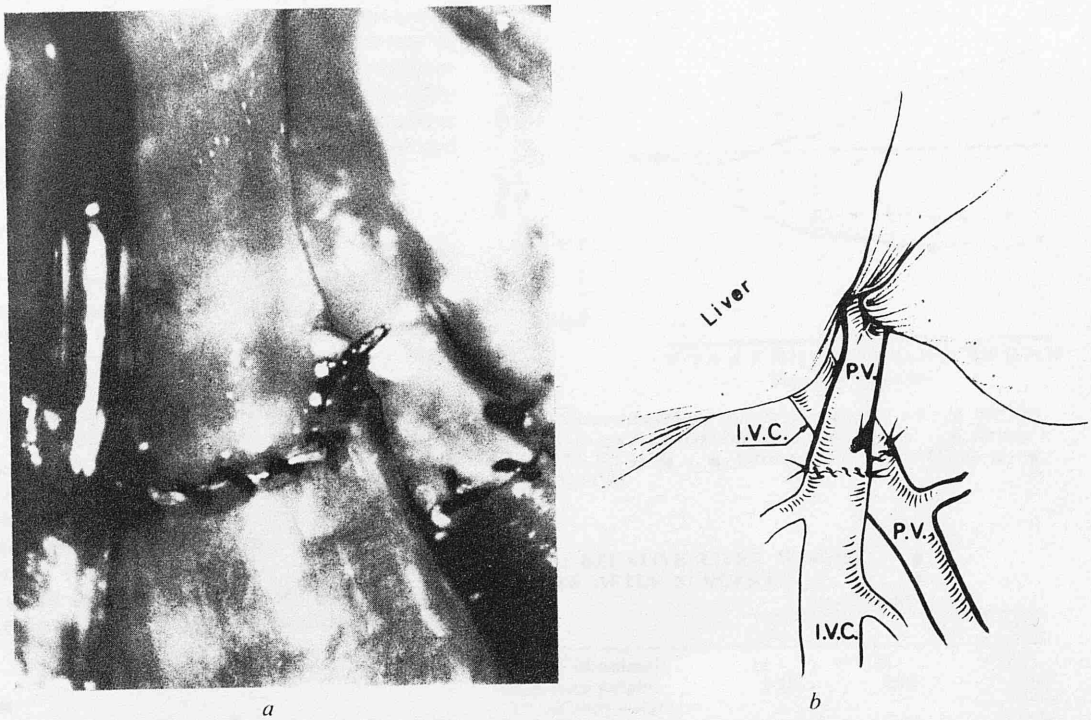
Fig. 2. Suturing of the proximal portal vein to the distal inferior vena cava (completed anastomosis, clamps in place). Note the importance of stay sutures.



a *b*

Fig. 3. Completed portacaval transposition.

because of wound infection, and 3 remain alive at present 150 days after operation.

A further 48 animals were studied. These were divided into the following groups:

Group 1 (13 animals): Portacaval transposition (PCT).

Group 2 (11 animals): End-to-side portacaval anastomosis (PCS) by a method similar to that of Lee and Fisher (1961).

Group 3 (14 animals): Sham operation in which the liver was mobilized and vessels displayed as in groups 1 and 2. Tissues were handled over a period of 40 minutes, but no vessels were clamped.

Group 4 (3 animals): Sham operation as in group 3 but with temporary clamping of vessels to simulate PCT.

Group 5 (3 animals): Sham operations as in group 3 but with temporary clamping of vessels to simulate PCS.

Group 6 (4 animals): PCT as in group 1 but with permanent ligation of the distal vena cava to the proximal portal vein anastomosis. In 2 animals this was performed immediately after operation, and in 2 animals 133 days after operation.

Groups 1–5 were studied over a 35-day period.

All the animals were weighed twice weekly. At the time of sacrifice the animals were anaesthetized with ether and the anastomoses examined. The liver was then excised, blotted, weighed and fixed in 10 per cent formol saline. In 5 animals from each of groups 1 and 2 and 5 control animals paraffin sections were stained with haematoxylin and eosin.

Patency of anastomoses

All the portacaval shunt and portacaval transposition anastomoses were examined before sacrifice and found to be patent. There was no evidence of thrombosis, stenosis of the anastomotic site or distension of the vessels distally. Adhesions around the operative field were found in every case but there was no evidence of collateral circulation, although this was not confirmed by splenoportography.

Changes in body weight (*Fig.* 4)

Animals in every group lost up to 18 per cent of their preoperative weight in the first week. Thereafter the sham-operated animals (groups 3–5) gained weight rapidly.

Rats with a portacaval shunt (group 2) continued to lose weight and by the third post-operative week had lost 13–32 per cent of their body weight. Recovery of this weight was minimal by 35 days. These weight changes were not related to the age of the animal.

Rats with a portacaval transposition (group 1) showed a body weight response to the operation which was related to the preoperative body weight (and thus to age). Younger rats of 270–325 g (6 animals) followed the trend of the sham-operated animals, though they remained significantly lighter ($P < 0{\cdot}001$) 35 days after operation. By contrast, older rats of 340–405 g (5 animals) lost more weight (12–20 per cent) by the end of the second week and thereafter their weights remained unchanged for most

of the period of study. However, by the thirty-fifth postoperative day these older rats with portacaval transposition did show some evidence of recovery of body weight and were at this time significantly heavier than the rats with portacaval shunts ($P = 0.05$). Only 2 animals with portacaval transposition did not behave in this manner, and these animals have been omitted from the calculations used for *Fig.* 4.

Liver weight and histology

The liver weight in each animal at sacrifice was expressed as a percentage of the body weight (*Table I*). In the control groups this was 3·0 per cent. The rats with portacaval transposition showed a relative decrease of liver weight to 2·78 per cent of body weight at sacrifice, which differed significantly from the control groups ($0.01 > P > 0.005$). This figure was the same for both age groups of rats described above. The animals with a portacaval shunt showed a significantly ($P < 0.001$) greater decrease in the relative liver weight at sacrifice (2·09 per cent) when compared with the animals with portacaval transposition.

Preliminary histological studies by light microscopy revealed only minimal red cell congestion of the sinusoids in the group with portacaval transposition.

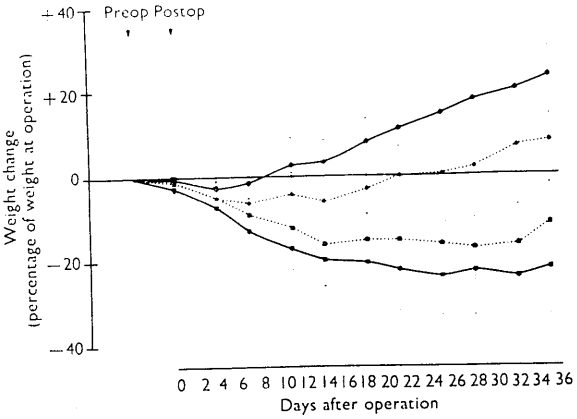


Fig. 4. Mean percentage weight change (± 1 s.d.) of the rats. ● — ●, Groups 3–5 (sham-operated controls). ● ... ● Group 1 (PCT, 270–325 g). ■ ... ■, Group 1 (PCT, 340–405 g). ■ — ■, Group 2 (PCS).

Table 1: RELATIVE LIVER WEIGHT 35 DAYS AFTER SURGERY

	Group		
	1	2	3–5
Number of animals	13	11	20
Mean liver weight (% of body weight)	2·78	2·09	3·00
Standard deviation	0·46	0·24	± 0·18

Discussion

We have shown that it is possible to perform portacaval transposition by simple direct end-to-end vascular anastomoses in the rat. The operation is short, well tolerated, and has a negligible mortality. Five animals have survived in a good state of health for 140–150 days after surgery.

It is known that acute occlusion of the portal vein in the rat is incompatible with survival. The 2 animals (group 6) in which the distal vena cava was ligated at the anastomotic site immediately after operation died within 48 hours. Since normal rats will survive ligation of the inferior vena cava above the renal veins (Franco et al., 1971), these animals were presumably unable to withstand the combined procedure. The 2 animals in which the vena cava was ligated at 133 days after operation survived the procedure but lost weight. This is compatible with the fact that the preparation has in effect been converted to a portacaval shunt. This procedure does not therefore adduce any evidence for patency of this anastomosis or existence of a collateral circulation further to that of inspection at sacrifice.

Portacaval transposition has a considerable theoretical advantage over other shunting procedures in the maintenance of perfusion of the liver via the portal vein. Whether the portal vein normally carries substances which are in some way trophic to the liver parenchyma is controversial (Fisher et al., 1971), but it seems certain that maintenance of blood flow via this route exerts a protective influence on the liver substance (Weinbren, 1955). The minimal sinusoidal congestion seen in the rats with portacaval transposition in this series may be a reflection of this continuing perfusion, and certainly the relative loss of liver weight is less after this procedure than after shunting. It seems unlikely that a weight difference of the magnitude that we have observed is due to a difference in content of blood within the liver, and it has previously been shown that cell volume is reduced in the liver of rats with portacaval anastomosis (Weinbren et al., 1972).

The reason for the age- or weight-related trends in body weight in the group with portacaval transposition is not immediately apparent, and it may be that longer periods of study are necessary to establish the validity of this observation.

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THE EFFECTS OF PORTACAVAL SHUNTING AND PORTACAVAL TRANSPOSITION ON SERUM IgG LEVELS IN THE RAT

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In rats subjected to end-to-side portacaval shunt and to portacaval transposition, serum IgG levels rose progressively by approximately 100% over a 5-week period. During the same period, sham-operated control animals showed only the increase expected with age. Rats with a portacaval shunt showed a greater fall in body weight and liver weight than did those with a portacaval transposition, and also showed a greater fall in levels of liver-synthesized proteins. Serum enzyme levels were markedly elevated during the first 48 hr after portacaval shunting, whereas after portacaval transposition the elevation was very small. Over the following 5 weeks enzyme elevations continued to be marginally greater in the portacavally shunted animals. Because IgG levels rose to a similar degree in both groups of animals, the present results support the hypothesis that hypergammaglobulinemia is due to the shunting of antigen-rich portal blood past the reticuloendothelial cells of the liver, and that hepatocellular damage does not play a major role in this process. The etiology of hypergammaglobulinemia in chronic liver disease in man may be similar.

The etiology of hypergammaglobulinemia in chronic liver disease is uncertain and the hypergammaglobulinemia appears to be largely independent of the nature of the liver disease. It may be presumed that elevated IgG levels are a result of increased antigenic stimulation, increased lymphoid reactivity, or decreased IgG catabolism. Although the results of investigations have been conflicting, most evidence now favors the first of these mechanisms. It has been shown, moreover, that the principal determinant of "normal" γ-globulin levels is stimulation by intestinal bacterial antigen[1] and several groups have now demonstrated an increase in antibodies to dietary and intestinal bacterial antigens in liver disease.[2-4] End-to-side portacaval anastomosis in experimental animals has been used to investigate this phenomenon by allowing the venous drainage of the intestine to bypass the liver.[5] The conventional portacaval anastomosis, however, suffers from the defects of producing a degree of liver atrophy, deterioration in the general condition of the animal, and evidence of hepatocellular dysfunction. Portacaval transposition achieves the same degree of portal-systemic shunting, but by the maintenance of better hepatic perfusion avoids some of these problems.[6] This paper describes changes in the serum levels of IgG (γ_2-globulin) and the liver-derived serum proteins albumin and slow α_1-globulin in the rat over a 5-week period after portacaval shunt, portacaval transposition, or sham operation.

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Materials and Methods

Male Sprague-Dawley rats weighing between 270 and 405 g were used. All of the procedures described were clean but not sterile, and no antibiotics were administered. Postoperatively the animals were caged in groups of 4 under controlled conditions of temperature and humidity and fed ad libitum on a standard laboratory rat cube diet (Diet 41B; Herbert Styles Ltd., Bewdely, Yorkshire, England).

A total of 44 animals was studied. They were divided into the following operative groups:

1. Portacaval transposition (PCT) by the method of Ryan et al.[6]—13 animals.
2. End-to-side portacaval anastomosis (PCS) by a method similar to that of Lee and Fisher[7]—11 animals.
3. Sham operation, in which the liver was mobilized and the vessels were displayed as in the first two groups. Tissues were handled intermittently over a period of 40 min and in some of the animals the portal vein and vena cava were temporarily clamped for a period similar to that required for performance of the PCT or PCS. It was subsequently found that those control animals whose vessels had been clamped behaved in the same way as those whose vessels had not been clamped, so that all were regarded as one control group—20 animals.

All of the animals were killed at 5 weeks. At this time the abdomen of each animal was opened and the anastomoses were inspected, before the animal was killed. All of the anastomoses thus examined were found to be patent.

Under ether anesthesia, blood was withdrawn from each animal preoperatively and at weekly intervals postoperatively by puncture of the retroorbital venous plexus. Serum was prepared by centrifugation and stored at −20°C until assayed. Assay of serum proteins was carried out using a radial immunodiffusion technique.[8, 9] The concentration of serum protein for each postoperative week was expressed as a percentage of the level found in the preoperative sample. To

minimize the effect of interplate variation, samples from any one rat were always measured on the same assay plate.[10] Specific rabbit antiserum to slow α_1-globulin was prepared as previously described.[11] Antisera to IgG and albumin were raised by immunizing rabbits with ammonium sulfate-precipitated IgG eluted from a diethylaminoethylcellulose column with 0.01 M phosphate buffer (ph 8.0), and with ammonium sulphate-precipitated albumin eluted from a carboxymethyl cellulose column with linear gradient 0.5 to 0.04 M acetate buffer (*p*H 5.0).

Results

General. There was no operative mortality and all animals survived the 5 weeks of study. The details of effects on body weight and liver weight have been reported previously.[6] These results are summarized in tables 1 and 2. It can be seen from table 1 that rats in the PCT group maintained their body weight significantly better than those in the PCS group by 5 weeks after surgery, although both groups were lighter than the control animals. Table 2 shows that there was a significant loss of liver weight in both groups but that animals in the PCS group showed a significantly greater fall in relative liver weight than those in the PCT group.

Serum proteins (table 3). Data are not available on all of the animals studied, because of insufficiency of serum, and the results described refer to estimations from the following numbers of animals: control—18 to 19, PCT—10 to 11, PCT—11.

There was a fall in the serum *albumin* levels in all three groups, although this was transient in the control group, and the percentage fall achieved statistical significance in only the PCS and PCT groups. The levels of albumin in the PCS group were significantly different from control animals at all time points, but animals with PCT showed a significant difference from control animals at the 4th and 5th weeks only. Despite this, there was no significant difference between the albumin levels of the PCS group and the PCT group at any one time point. However, if data for the first 3 weeks are pooled for each of these two groups, giving in each case the mean of 33 observations in 11 animals, we obtain a mean percentage fall in the albumin level of 20% (± 23) for the PCT group and 30% (± 18) for the PCS group, the difference between the means being significant ($P < 0.05$).

Slow α_1-globulin. This rose postoperatively in all three groups. In the control group and in the PCT group this rise was maintained throughout the period of study and at no time did these two groups differ significantly. The PCS group, however, showed a progressive decline from the initial elevated level found at the 1st week, and by the 4th and 5th weeks had fallen below the preoperative level. At the 4th and 5th weeks the PCS group differed significantly from both the control group and the PCT group.

TABLE 1. *Mean body weight of rats 5 weeks after PCS, PCT, or control procedure*

Group	Body wt as % preop wt (mean ± 1 SD)[a]
Control (20 rats)	122.9 ± 6.9
PCT (13 rats)	99.3 ± 10.9
PCS (11 rats)	78.9 ± 11.6

[a] All three pairs of groups differ significantly, $P < 0.0005$.

TABLE 2. Relative liver weight of rats 5 weeks after PCS, PCT, or control procedure

Group	Liver wt as % body wt when killed (mean ± 1 SD)
Control (20 rats)	2.99 ± 0.17
PCT (13 rats)	2.77 ± 0.23[a]
PCS (11 rats)	2.12 ± 0.24[b]

[a] Significantly different from control, $P < 0.0025$.
[b] Significantly different from PCT, $P < 0.0005$.

TABLE 3. *Serum protein levels (as % of preoperative level) after PCS, PCT, or control operation (mean + 1 SD)*

Serum protein	Weeks postop 1	2	3	4	5	
Albumin						
Control	92 ± 22	95 ± 25	93 ± 23	105 ± 25	104 ± 22	
	(<0.02)	(<0.001)	(<0.025)	(<0.025)	(<0.005)	(*P** control vs PCS)
PCS	74 ± 15	63 ± 19	74 ± 18	81 ± 25	79 ± 20	
PCT	79 ± 24	77 ± 27	82 ± 20	80 ± 14	86 ± 20	
				(<0.005)	(<0.05)	(*P** control vs PCT)
Slow α_1 globulin						
Control	117 ± 15	119 ± 17	113 ± 14	113 ± 18	118 ± 22	
				(<0.02)	(<0.005)	(*P** control vs PCS)
PCS	115 ± 14	112 ± 9	107 ± 11	99 ± 12	93 ± 16	
				(<0.005)	(<0.02)	(*P** PCS vs PCT)
PCT	118 ± 16	107 ± 13	116 ± 11	118 ± 14	112 ± 15	
IgG						
Control	88 ± 30	111 ± 30	115 ± 33	126 ± 30	139 ± 45	
		(<0.005)	(<0.001)	(<0.005)	(<0.01)	(*P** control vs PCS)
PCS	93 ± 27	156 ± 39	198 ± 62	192 ± 57	197 ± 60	
PCT	113 ± 38	146 ± 52	188 ± 58	208 ± 65	224 ± 67	
		(<0.05)	(<0.001)	(<0.001)	(<0.001)	(*P** control vs PCT)

*P** = *P* value for differences between groups compared using Student's *t*-test.

IgG. In control animals this rose by 39% by the 5th postoperative week. In the PCS and PCT groups, however, the levels rose much more rapidly, increasing by 97% and 124%, respectively, by the 5th postoperative week. There was at no time a significant difference between the PCS and PCT groups, but both groups differed significantly from the control animals at all points from the second week onward.

Liver histology. As previously reported,[6] light microscopic examination of H & E-stained sections of liver from animals in all groups revealed no morphological abnormalities, and a "blind" observer was unable to allocate the sections to operative groups. The only histological change found on further examination with prior knowledge of the groups was a slight degree of sinusoidal congestion in the livers of animals with PCT.

Serum enzyme levels. Changes in liver-associated enzymes (SGOT, SGPT, alkaline phosphatase, lactate dehydrogenase, and creatine kinase) have been measured in the sera obtained in this and other experiments, the results of which will be reported in full separately. In summary, however, the levels of most enzymes showed a modest elevation (up to twice the control level) during the 5 weeks after PCS and PCT. Although levels after PCS were higher at most time points than after PCT, only in the case of SGPT did this difference achieve statistical significance. When enzyme levels were measured at intervals up to 48 hr in a similar experiment, it was found that there was a very high transient elevation of SGOT and SGPT after PCS, whereas after PCT the changes did not differ from those seen in control animals (I. S. Benjamin, C. J. Ryan, S. Caine, and L. H. Blumgart, *unpublished observations*).

Discussion

The present data confirm the results of Keraan et al.[5] that serum IgG levels are increased after PCS in the rat. This group also demonstrated elevated titers of antibody to specific intestinal bacterial antigens. Estimations of such titers is now in progress in this laboratory. Keraan et al.'s study over a 12-week period showed that IgG levels continued to rise slowly beyond the period of 5 weeks which these authors have examined. In addition to this it has been shown in this laboratory that IgG levels behave in a similar fashion after PCT in the rat. The degree of elevation which has been found here after control operation has been shown previously to be consistent with normal age changes for animals in this weight range.[12] The authors feel that this result is of special interest, because results from this and from other laboratories suggest that hepatocellular function is maintained better after PCT than after PCS.

It has been shown elsewhere that in the dog hepatic blood flow and clearance of bromosulfophthalein and galactose are greatly reduced after PCS, but are maintained well after PCT.[13-16] Studies in progress in this laboratory suggest that these results will be confirmed in the rat (I. S. Benjamin et al., *unpublished observations*). It has been demonstrated here that serum enzyme levels are significantly higher after PCS than after PCT, especially during the first 48 hr after surgery. Levels of the liver-derived proteins albumin and slow α_1-globulin are lower after PCS than after PCT, although in the case of albumin the difference is of borderline significance. It has also been noted here and elsewhere that after PCT in the rat the body weight falls to a lesser degree and is more rapidly regained than after PCS,[6, 17] and that the fall in liver weight is also significantly less after PCT.[6] These authors have not demonstrated a histological counterpart to the fall in liver weight, but other studies on PCS suggest that measurement of cell size may produce such information.[18]

The nature of the hypergammaglobulinemia seen in experimental preparations of this type has been the subject of considerable debate. Havens et al.[19] proposed a generalized enhanced responsiveness to antigenic stimuli in liver disease, whereas Popper and his co-workers[20-22] suggest that intrahepatic reticuloendothelial cells may produce a significant amount of γ-globulin under pathological conditions. More recently, Starzl[23] has shown that γ-globulins with the genotypic characteristics of the donor have been found in the sera of recipients of liver transplants.

However, the Kupffer cells are usually said to lack the ability to process antigen for an antibody response,[24] and it has been proposed that the physiological role of these cells is simply to act as a filter for intestinal dietary and bacterial antigen and to deny such antigen access to the immunologically competent cells of the reticuloendothelial system. Failure of one manifestation of this sequestering function (the Chase-Sulzberger effect) has been demonstrated after portacaval transposition in the dog.[25] Such failure in liver disease may be due to direct damage to the Kupffer cells or to their saturation by phagocytosis of the breakdown products of liver damage, or it may be due to bypassing of these cells through portal-systemic shunts, either intra- or extrahepatic.[26]

The role of portal-systemic shunting relative to hepatocellular damage as a causative factor in the hypergammaglobulinemia of chronic liver disease remains unclear in the human situation. In the present report it has been shown that the changes in IgG levels after PCS and after PCT are closely similar. If elevation of IgG were closely linked to hepatocellular damage and to disturbed liver function, one might expect to see a significant difference between the two groups, because there is good evidence that liver function is significantly more impaired after PCS than after PCT. The fact that such a difference is not observed suggests that the chief determinant of the elevated IgG levels in this model is portal-systemic shunting rather than hepatocellular dysfunction.

It is suggested that portacaval transposition in the rat as an experimental preparation for the study of these immunological phenomena may prove to have some advantage over conventional portacaval shunting. The present results suggest that the prime factor responsible for the hypergammaglobulinemia of liver disease may be portal-systemic shunting per se with hepatocellular damage playing a less significant role.

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PORTACAVAL TRANSPOSITION AND SUBSEQUENT PARTIAL HEPATECTOMY IN THE RAT: EFFECTS ON LIVER ATROPHY, HYPERTROPHY AND REGENERATIVE HYPERPLASIA

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Summary.—Portacaval transposition diverts portal blood from the liver. It allows systemic venous blood to perfuse the portal bed.

Body weight and liver weight have been followed before and after portacaval transposition and control procedures in rats, and the DNA activity ratio studied in the liver of rats after partial hepatectomy in portacavally transposed animals.

The results suggest that the liver atrophy seen after portal diversion is a result of diversion of trophic substances in the portal blood rather than of a decrease in absolute liver flow.

Recovery of liver weight after partial hepatectomy in portacavally transposed animals occurs within the same time as in control animals, and the time course and magnitude of regenerative hyperplasia, as assessed by liver DNA activity ratio, is unimpaired.

PORTACAVAL SHUNT is followed by profound changes in liver size and function, but there has been difficulty in determining whether these changes are due to alterations in the total quantity of blood supplied by the portal vein to the liver or to diversion of specific constituents within the portal blood. In addition, the difficulty in separating the effects of diversion of portal blood from the changes consequent on resection of liver mass has been an obstacle in the investigation of liver regeneration in the portally deprived animal.

Recently it has been suggested that diversion of endogenous insulin is responsible for the atrophy after portal shunt (Starzl *et al.*, 1975) and intraportal exogenous insulin has been shown to ameliorate this atrophy (Starzl *et al.*, 1976). In addition, it is proposed that the contents of portal blood, and insulin in particular, may be of importance in the process of regenerative hyperplasia (Starzl *et al.*, 1975; Starzl *et al.*, 1976).

Portacaval transposition diverts portal blood from the liver through the cephalad inferior vena cava. Systemic venous blood from the caudal inferior vena cava perfuses the portal bed maintaining the dual liver blood supply and total hepatic blood flow (Child *et al.*, 1953; Heer, Silvius and Harper, 1960; Starzl *et al.*, 1962; Kreutzer and Schenk, 1971). Liver size and function after partial hepatectomy may thus be studied in the presence of adequate hepatic blood flow without direct perfusion of the liver by blood flowing from the intestines and pancreas.

Much experimental work related to liver regeneration has been done in the rat and the proliferative response which follows resection is well documented (Bucher, 1963). This study records the changes in the liver/body weight ratio which follow end-to-end portacaval transposition in the rat (Ryan, Benjamin and Blumgart, 1974). In addition, the regenerative hyperplastic response consequent upon partial hepatectomy is examined in rats subjected to portacaval transposition.

MATERIAL AND METHODS

1. *Animals.*—Male Sprague–Dawley rats weighing between 200–220 g were fed on a standard pellet diet (41 b Oxoid Ltd), allowed water *ad libitum* and housed in a constant temperature and controlled 12-h daylight environment.

2. *Surgical procedures.*—All surgical procedures were carried out under open ether anaesthesia through a midline ventral incision.

Portacaval transposition was performed by the method of Ryan *et al.* (1974). Immediately prior to portacaval transposition, a left adrenalectomy was performed since, in the rat, the left adrenal gland venous effluent drains *via* the left renal vein into the inferior vena cava. This was done because glucocorticoids have been shown to inhibit mitotic activity in the liver (Hyde and Davis, 1966) and following portacaval transposition, inferior vena cava blood drains directly to the liver.

Adrenalectomy was performed by simple excision after ligation of the adrenal vessels. In animals in which adrenalectomy alone was performed, the liver was not disturbed.

Sham portacaval transposition was performed by dissection of the vessels as for portacaval transposition and the portal vein and inferior vena cava were clamped for similar time periods.

Partial hepatectomy was carried out by the method of Higgins and Anderson (1931). In order to avoid variation in the mitotic activity due to diurnal rhythm (Jaffe, 1954), partial hepatectomy and control procedures were performed between the hours of 9 a.m. and 11 a.m.

The abdominal wound was closed with continuous catgut for the muscle layers and stainless steel clips to the skin.

3. *Relative liver weight.*—The animals were weighed before operation and at intervals thereafter. On killing, 3 or 6 weeks after the initial procedure, the abdomen was opened and the portal vein clamped. The liver was removed, blotted and weighed.

Liver weight was expressed as a percentage of body weight (relative liver weight). The normal value ($\pm$ s.d.) for this ratio was first obtained in a separate group of 5 animals and found to be $4{\cdot}23 \pm 0{\cdot}29\%$. Comparison of this ratio between groups was made by Student's t test incorporating a Fisher's F test of comparison of variance.

4. *Measurement of DNA synthesis.*—DNA synthesis was measured by the method of Weinbren and Woodward (1964) with a modification for the use of tritiated thymidine (Weinbren, Arden and Stirling, 1969). Each animal in each group received 100 μCi of tritiated thymidine (Radiochemical Centre, Amersham: specific activity 20,000 μCi/mmol) by injection into the jugular vein 1 h before killing.

Radioactivity in the DNA extracted from the liver was measured in a Packard Tricarb liquid scintillation counter and corrected to dpm by a linear quench calibration curve based on the channel ratio. The radioactivity of the DNA in dpm is expressed as a ratio of the total DNA estimated by measurement of its optical density in a SP 600 spectrophotometer at 260 nm wave length.

The DNA activity ratio was statistically analysed by the Mann-Whitney non-parametric test utilizing Wilcoxon's U statistic.

5. *Arrangement of experiments.*—Experiments are reported in two parts, utilizing the following groups of animals:

Group I (experimental group)
Portacaval transposition + left adrenalectomy (PCT + A)
Group II (control group)
Sham portacaval transposition + left adrenalectomy (Sham + A)
Group III (control group)
Left adrenalectomy alone (Ad)
Group IV (control group)
(Experiment 2 only)
Ether anaesthesia alone (Eth)

Experiment 1

In this experiment the relative liver weight was measured in animals after portacaval transposition plus left adrenalectomy and control procedures.

Fifteen of 16 animals survived PCT + A (Group I) and 3 weeks later one-third of the animals was killed, the anterior and left lateral lobes being removed as for partial hepatectomy, and weighed. The remnant of the liver was then removed and similarly weighed, and the total liver weight noted. A further one-third of the animals was subjected to partial hepatectomy but allowed to survive a further 3 weeks (6 weeks from the time of portacaval transposition). They were then killed and the liver remnant weighed. The remaining animals were not subjected to partial hepatectomy but allowed to survive 6 weeks and were then killed and the liver removed and weighed.

Fifteen rats (Group II) survived sham portacaval transposition + adrenalectomy, and 10 left adrenalectomy alone (Group III). The animals in Groups II and III were treated in an identical manner to those in Group I, one-third of the animals in each group being killed at 3 weeks, one-third receiving partial hepatectomy, and one-third being allowed to survive until the 6th postoperative week without partial hepatectomy.

Experiment 2

In this experiment, the DNA synthetic activity was measured at 8 time points during

the first 72 h following partial hepatectomy. Within each of the experimental groups I, II and III, and for each of the time points studied, batches of rats (4–7 rats per batch) were subjected to the initial operation (portacaval transposition or control operation) and were allowed to recover and survive for 3 weeks, at which time partial hepatectomy was performed. The animals were allowed to survive and a batch of animals was killed at each of the time points, 12, 18, 21, 24, 30, 36, 48 and 72 h after partial hepatectomy. Duplicate batches were assessed for the time intervals 18, 21 and 24 h in order to cover the known peak of DNA synthetic activity associated with hepatocyte replication (Bucher, 1963).

In addition, for each batch of animals at each time interval, 2 further control animals (Group IV—Eth) were used. These 2 animals were anaesthetized at the time of the initial operation and then 3 weeks later one animal was subjected to a further ether anaesthetic and one to sham partial hepatectomy, the liver being mobilized but no resection carried out.

Each animal in each group received 100 μCi of tritiated thymidine 1 h before killing by intravenous injection.

At sacrifice a 700–800-mg portion of the right lateral lobe was deep-frozen for later estimation of DNA synthetic activity.

RESULTS

Two hundred and fifty rats were used in the 2 experiments and 218 (87%) survived to be killed. In animals subjected to PCT + A (Group I), 78% survived and 87% of animals in Group II (Sham + A) survived. In those animal groups subjected to adrenalectomy alone (Group III) or ether anaesthesia (Group IV), survival was 96%.

Animals in all groups gained weight after the initial operative procedure (Table I). However, portacavally transposed animals did not gain as much weight as did control groups (PCT + A *vs* Sham + A, $P < 0{\cdot}001$).

TABLE I.—*Body Weight at Initial Procedure and at Partial Hepatectomy*

Group	No.	Initial body weight	Body weight at partial hepatectomy
I (PCT + A)	58	210·2±6·9	265·3±29·5
II (Sham + A)	68	210·2±6·7	289·5±26·9
III (Ad)	50	210·7±5·5	303·6±31·5
IV (Eth)	25	209·3±7·1	316·8±26·8

PCT + A *vs* Sham + A, $P < 0{\cdot}001$ at partial hepatectomy.

Following partial hepatectomy, the animals in all groups lost weight. This loss of weight was significant at 3 weeks after partial hepatectomy in Group I ($0{\cdot}05 > P > 0{\cdot}025$) and Group II ($0{\cdot}01 > P > 0{\cdot}005$) but not in Group III.

Experiment 1

Table II shows the changes in relative liver weight in the 6 weeks after portacaval transposition or control operation. A progressive reduction in the relative liver weight was found in all animals in all groups not submitted to partial hepatectomy but this reduction was significantly greater at 3 and at 6 weeks in animals subjected to PCT + A (Group I) as compared to sham operation (Group II) ($0{\cdot}005 > P > 0{\cdot}001$) at 3 and at 6 weeks).

In animals subjected to partial hepatectomy 3 weeks after the initial procedure and then immediately killed, it was found that the relative weight of the liver remnant (after partial hepatectomy) was not significantly different in any of the

TABLE II.—*Experiment 1—Relative Liver Weight*

	At 3 weeks after initial procedure		At 6 weeks after initial procedure	
Group	Total	Remnant after partial hepatectomy	No partial hepatectomy	Partial hepatectomy
I (PCT + A)	3·29±0·19	1·26±0·19 (5)	2·88±0·23 (5)	2·47±0·42* (5)
II (Sham + A)	3·96±0·32	1·45±0·17 (6)	3·64±0·13 (4)	3·47±0·37* (5)
III (Ad)	3·77±0·22	1·26±0·12 (4)	3·31±0·28 (3)	3·35±0·46* (3)

* No significant difference compared to non-hepatectomized group at 6 weeks ($P > 0{\cdot}05$).
Normal value of relative liver weight determined in 5 rats = 4·23 ± 0·29.
Figures in parentheses = no. of animals.

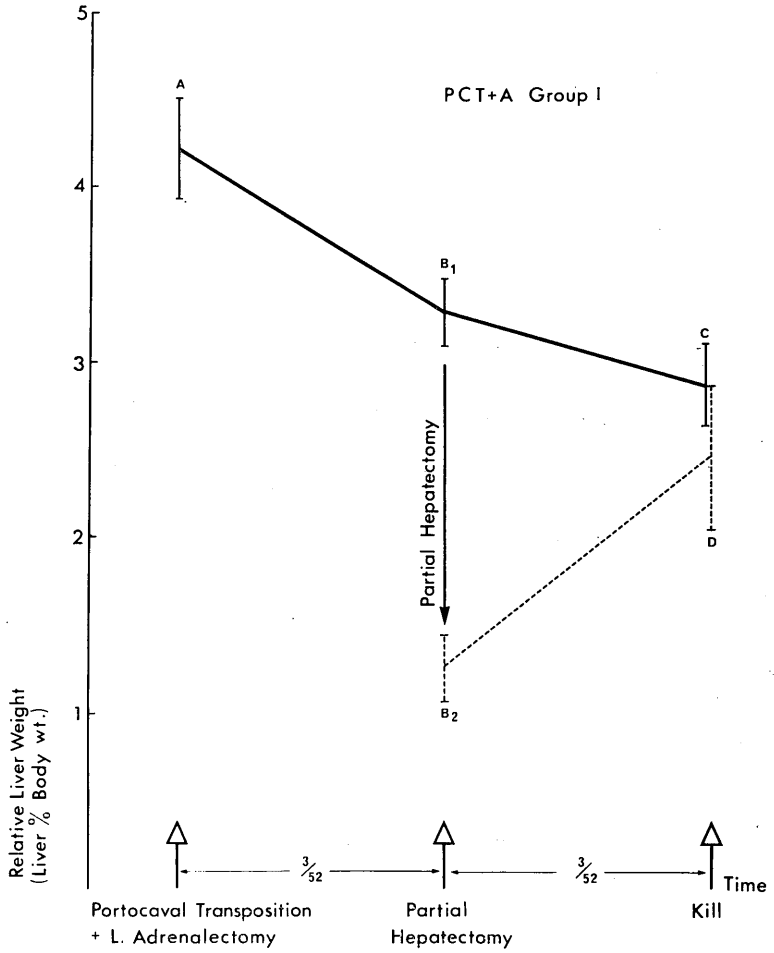

FIG.—Changes in relative liver weight (Experiment 1). A–B_1–C, Liver atrophy following PCT + A. B_2, Relative weight of liver remnant after partial hepatectomy. B_2–D, Gain in relative weight of remnant after partial hepatectomy in PCT + A animals. CD, $P > 0{\cdot}05$. No significant difference. Each point A, B, C and D represents the mean ± s.d. at time of killing for a separate group of 5 animals (see text).

groups. The relative liver weight 6 weeks after the initial procedure (3 weeks after partial hepatectomy) was significantly less in animals in Group I when compared to animals in Groups II or III, but it did not differ significantly from the expected relative liver weight found in non-hepatectomized animals of Group I 6 weeks after the initial portacaval transposition (Table II, Fig.). The changes in relative liver weight in animals of Group I (PCT + A) reveal that the atrophy consequent on a portacaval transposition results in a smaller liver 9 weeks after operation. However, in animals subjected to partial hepatectomy during this period, there is recovery to expected liver weight in the same time period as for control animals without portal diversion (Table II).

Experiment 2

Examination of the DNA activity ratio after partial hepatectomy in Groups I, II and III revealed a rise to peak DNA synthetic activity 21 to 24 h after partial

TABLE III.—*Experiment 2—DNA Activity Ratio after Partial Hepatectomy (P. H.) (Median Results)*

Group	12 h	18 h	21 h	24 h	30 h	36 h	48 h	72 h
I (PCT + A)	345 (5)	10461 (8)	23101 (10)	14592 (5)	4981 (4)	8116 (5)	3533* (3)	8540* (4)
II (Sham + A)	290 (5)	10590 (12)	21635 (8)	24079 (11)	11281 (5)	10547 (6)	12717 (6)	2778 (6)
III (Ad)	517 (3)	34096 (7)	32324 (8)	26495 (7)	8059 (4)	17689 (4)	11216 (4)	8797 (4)
IV (Eth)	354 (2)	286 (4)	378 (3)	548 (4)	354 (1)	214 (2)	323 (2)	450 (2)

* Significant difference when compared to Group II (Sham + A) ($P < 0{\cdot}05$).
Figures in parentheses = no. of animals killed.

hepatectomy, whereas in Group IV (ether controls, no partial hepatectomy) no such rise occurred (Table III). Statistical comparison between Group I (PCT + A) and Group II (Sham + A) at each time point from 12–36 h after partial hepatectomy revealed no statistical difference, although at time points 48 and 72 h, by which time the greater part of hepatocyte DNA synthetic activity is complete, there was a significant difference ($P < 0{\cdot}05$) detectable (Table III).

DISCUSSION

Ever since Mann (1944) suggested, on the basis of observations of changes in liver weight, that the regenerative response following partial hepatectomy was diminished by deviation of portal blood, there has been difficulty in deciding upon the role of the portal circulation in the control of liver regeneration. It has, however, been demonstrated that, although portacaval shunt or indeed portal venous ligation to a segment of liver results in atrophy of liver tissue, the capacity for a regenerative hyperplastic response following partial hepatectomy is retained (Weinbren, 1955; Fisher *et al.*, 1962; Weinbren *et al.*, 1972). Indeed, partial hepatectomy is not necessary for the initiation of a proliferative response, since the cellular atrophy which occurs after portal venous ligation represents a net loss of liver tissue, and this in itself may act as a stimulus sufficient to initiate a hyperplastic response (Weinbren *et al.*, 1975).

Child (1953) utilized the operation of portacaval transposition to explore liver regeneration in the dog and suggested that the operation allowed recovery of liver size after partial hepatectomy even though portal blood was entirely deviated. More recently, Lee *et al.* (1974) reported recovery of liver size after partial hepatectomy in portacavally transposed rats. However, their studies of regenerative hyperplasia relied on measurements of DNA synthesis made as late as the 7th day after partial hepatectomy and, since this is well beyond the peak of hepatocyte replication (Bucher, 1967), the results must be interpreted with caution.

Not only the weight of the liver but the weight of the animal changes after operation. Expression of liver weight as a reflection of atrophy or of recovery after partial hepatectomy must take this into account. The results presented confirm our earlier observations (Ryan *et al.*, 1974) that all animals in all groups regained weight after the initial operative intervention but that portacavally transposed animals did not gain as much weight as control groups. In addition, portacaval transposition is associated with a degree of liver atrophy reflected by a fall in relative liver weight, which was evident by the 3rd postoperative week and was also progressive, so that 6 weeks after operation there was a significant further fall in the relative liver weight. Nevertheless, in animals in which partial hepatectomy was carried out 3 weeks after portacaval transposition, there was a recovery by the 6th week to the liver

weight previously defined in animals with portacaval transposition but not subjected to partial hepatectomy (Fig.). This recovery occurred within the same time period as did recovery of liver weight in the control animals subjected to partial hepatectomy without previous portacaval transposition.

The relationship of the atrophy consequent on portacaval transposition to the possible alteration in blood flow is of interest. Liver blood flow has been demonstrated to be at least as great as normal after portacaval transposition in the dog (Heer *et al.*, 1960; Starzl *et al.*, 1962; Kreutzer and Schenk, 1971). More recently, we have studied liver blood flow before and after partial hepatectomy in the rat, using an ^{85}Krypton clearance technique (Rice *et al.*, 1976) and have also demonstrated that total nutritional blood flow to the liver remains the same before and after portacaval transposition (Ryan *et al.*, in preparation). Although a greater loss of relative liver weight occurs after portacaval shunt than after portacaval transposition (Ryan *et al.*, 1974), and this may be flow related, the atrophy that we have demonstrated after portacaval transposition does not appear to be associated with a fall in total liver blood flow. Our observations support the suggestion that liver atrophy is a consequence of deviation of factors within the portal blood rather than the result of a decrease in total flow alone (Starzl *et al.*, 1975; Starzl *et al.*, 1976). However, it is of particular interest that, despite the atrophic changes occurring in the liver of portacavally transposed animals, we found no change in the DNA activity ratio, either in timing or degree, in the first 36 h after partial hepatectomy. This observation suggests that regenerative hyperplasia following partial hepatectomy is not impaired by diversion of portal blood factors. Whether the large increase in blood flow per gram of liver tissue following partial hepatectomy in normal rats (Ryan *et al.*, unpublished) is concerned with the initiation or is permissive of the process of DNA synthesis remains to be seen.

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CHANGES IN THE NUCLEI OF ASTROCYTES FOLLOWING PORTACAVAL SHUNTING AND PORTACAVAL TRANSPOSITION IN THE RAT

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Summary.—Structural abnormalities are found in the astrocytes of the dentate nuclei of animals after portacaval shunting (PCS). These changes are also found in man in association with portal–systemic encephalopathy. To investigate the relationship between portal–systemic shunting and hepatocellular dysfunction in the pathogenesis of these changes, PCS and portacaval transposition (PCT) were performed in rats. PCT diverts portal blood into the systemic circulation, but retains normal total hepatic blood flow by perfusion with systemic venous blood. Liver function and mass are better preserved than after PCS. Abnormal glial cells were found in 4·03% of animals following sham operation, 13·45% following PCT, and 19·09% following PCS. Both experimental groups differed significantly from control animals, and the number of abnormal cells was significantly higher after PCS than after PCT. These findings are in keeping with the hypothesis that hepatocellular dysfunction plays an important role in addition to portal–systemic shunting in the aetiology of the structural changes in the brain associated with hepatic encephalopathy.

ABNORMALITIES in astrocytes, which are associated with some forms of liver disease, were first described by von Hösslin and Alzheimer in 1912. The relationship of these changes to the clinical state of hepatic encephalopathy has not been completely elucidated, but there is wide acceptance of a broad hypothesis that they are due to the passage of unaltered constituents of portal venous blood to the systemic circulation. About 20% of patients develop hepatic encephalopathy after surgical portacaval anastomosis (Mutchnik, Lerner and Conn, 1974; Read *et al.*, 1968), and it has been suggested that this condition depends not only on portal–systemic shunting but also on concomitant hepatocellular dysfunction (Warren and Rebouças, 1964).

Of the abnormalities described by von Hösslin and Alzheimer (1912), that most often seen in patients with liver disease or portacaval anastomosis is an apparent enlargement and pale staining of astrocytic nuclei with varying degrees of folding and indentation of the nuclear membrane. This abnormality is known as the Alzheimer Type II change, and when fully developed is readily recognizable in routinely stained microscopical preparations from most of the grey matter areas of the brain. A proportion of glial nuclei, however, remain normal in appearance. The occurrence of similar changes in the nuclei of astrocytes after experimental portacaval anastomosis has been described in dogs (Kline *et al.*, 1966) and in rats (Doyle, 1967; Cavanagh and Kyu, 1969). Portacaval anastomosis in rats produces hepatic atrophy and functional deterioration (Lee *et al.*, 1974).

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In an attempt to explore the relationships of hepatocellular dysfunction and portal–systemic shunting to the development of the Alzheimer Type II astrocyte, the effects of end-to-side portacaval shunt (PCS), have been compared to those of portacaval transposition (PCT). The latter operation crosses the portal venous and inferior vena caval circulation, so that the portal blood is diverted to the systemic circulation as in the PCS, but hepatic perfusion is maintained by the flow of venacaval (systemic) blood through the portal vessels. This operation produces less atrophy of the liver and less severe hepatocellular dysfunction than PCS (Ryan, Benjamin and Blumgart, 1974; Benjamin *et al.*, 1976; Guest *et al.*, 1977).

MATERIALS AND METHODS

Animals.—Male Sprague–Dawley rats (250–400 g) were subjected to end-to-side PCS by the method of Lee and Fisher (1961; 5 animals), PCT by the method of Ryan *et al.* (1974; 9 animals), or a control procedure in which laparotomy was performed with dissection of the portal vein and inferior vena cava as for portacaval transposition and clamping of the portal vein for 15 min (6 animals). All operations were carried out under ether anaesthesia in clean but not sterile conditions. The animals were thereafter housed for 5 weeks under controlled conditions of temperature and humidity and fed *ad libitum* on a standard laboratory rat cube diet (Diet 41B, Herbert Styles Ltd, Bewdley, Yorkshire, England: nitrogen content 23·5 mg/g). Animals were weighed and blood was withdrawn at weekly intervals under ether anaesthesia by puncture of the retro-orbital venous plexus. At 5 weeks the animals were killed and the anastomoses inspected and shown to be patent before the animal was killed. Rapid retrograde cannulation of the aorta was carried out and the head perfused with formal acetic methanol (40% formalin: glacial acetic acid: absolute methanol—1:1:8) for several minutes.

Histological methods.—The brains were dissected and processed to paraffin in a routine manner. Seven-micron sections of cerebrum, brainstem and cerebellum were stained by standard techniques with haematoxylin and eosin, periodic acid–Schiff, cresyl violet, Palmgren's silver impregnation technique for outlining neuronal processes (Palmgren, 1960), and the luxol fast blue method for myelin.

Cytological examination.—Sections from each animal stained by each of the above techniques were scanned for abnormalities and, in the light of findings in previous studies (Doyle, 1967), particular attention was paid to the cerebellar dentate nuclei. Total counts of neurons and of normal and abnormal astrocyte nuclei were made in the dentate nucleus, selected brainstem nuclei, the basal ganglia and the cerebral cortex. The changes sought in the astrocyte nuclei were: (i) enlargement of the nucleus, (ii) excessive indentation and folding of the nuclear membrane, (iii) pale staining of the nucleus, and (iv) peripheral disposition of the stainable DNA (Figure 1). Comparison of astrocyte nuclear size was made by measurement of the diameter with a micrometer eyepiece.

RESULTS

Behaviour and body weight

There was no mortality in the group of animals studied. The general changes in these animals have been described previously (Ryan *et al.*, 1974). None of the animals exhibited abnormal behaviour or signs of encephalopathy.

There was an initial marked loss of weight in both experimental groups, which was rapidly regained in the animals with PCT, but persisted in the animals with PCS. Control animals gained weight normally after the first few days following laparotomy.

Liver weight and morphology

Changes in the relative liver weight at death (weight of liver as a percentage of body weight) have also been previously reported. There is a degree of liver atrophy following PCT at 5 weeks, but this is significantly less than that which follows PCS. No significant histological differences in the liver between the 2 groups were noted.

Biochemical studies

These have been previously reported (Benjamin *et al.*, 1976). The levels of SGOT and SGPT in the serum measured at weekly intervals showed a modest elevation after both PCS and PCT with significantly higher levels after PCS. During the first 48 h after PCS, but not

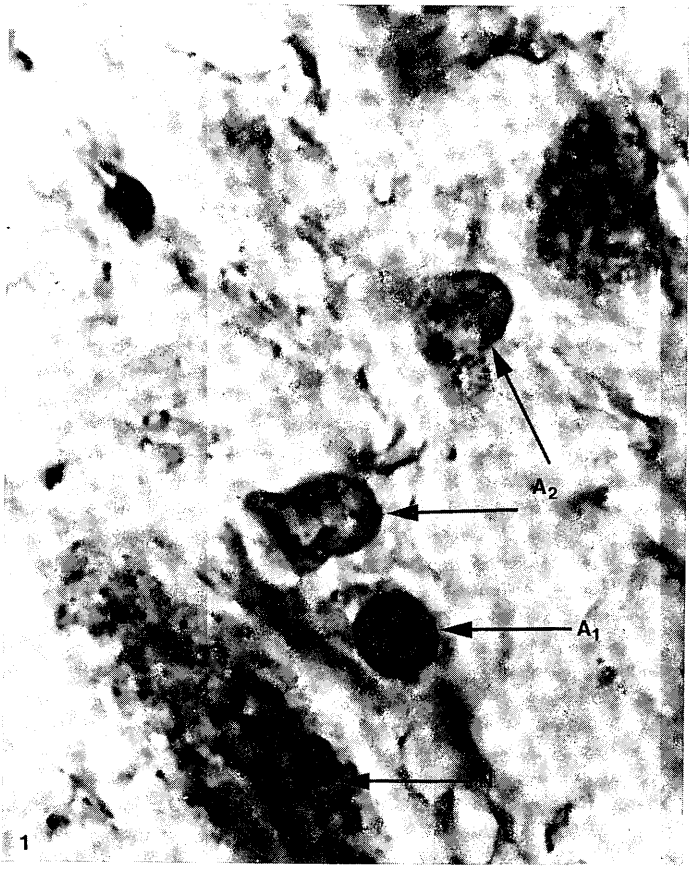

FIG.—Section of dentate nucleus in a rat 5 weeks after PCS (× 2500: stained luxol fast blue and cresyl violet). N—normal neurone, A1—normal astrocyte nucleus, A2—abnormal astrocyte nuclei, showing features of Alzheimer Type II change.

after PCT, very high levels of SGOT and SGPT were found. The liver-synthesized proteins albumin and α_1-globulin fell significantly more after PCS than after PCT.

Cytological changes

Astrocytic nuclei were measured in all areas of the brain using a micrometer eyepiece and no enlargement of nuclei was found in any of the sections.

Scanning of sections taken from cerebral hemispheres, cerebellum and brainstem showed in each area a small number of astrocytic nuclei with some or all of the criteria for abnormality, with the exception of nuclear enlargement. These changes were found in the cerebral cortex, central nuclei, brainstem nuclei and dentate nuclei of the cerebellum in normal and control animals and also in animals with PCS and PCT. Counting of normal and abnormal nuclei, however, showed no difference between normal, control and experimental animals in all areas of the brain except the dentate nucleus. It was concluded that in most areas of the nervous system a range of light microscopical appearances occurs in normal animals, and the PCS and PCT do not produce significant morphological nuclear changes in these areas.

In the dentate nucleus, however, there was a marked difference between the experimental and control animals. In each animal approximately 400 glial cells were evaluated and counted in the

TABLE.—*Abnormal Glial Cells in Dentate Nuclei (% of at least 400 cells)*

Control ($n = 6$)	PCT ($n = 9$)	PCS ($n = 5$)
3·97	12·50	12·75
3·25	17·40	17·70
1·98	12·70	23·00
6·40	16·96	27·00
3·00	11·97	15·00
5·60	9·50	
	13·00	
	15·90	
	11·10	
Mean 4·03 ± 1·67 (s.d.)	13·45*‡ ± 2·71 (s.d.)	19·09† ± 5·85 (s.d.)

* Significantly different from control, $P < 0·001$.
† Significantly different from control, $P < 0·002$.
‡ Significantly different from PCS, $P < 0·05$. (Student's t test)

dentate nuclei (Table). In the normal and sham-operated animals a mean of 4·03% of astrocytes showed nuclear abnormalities. In the PCS and PCT rats, the mean percentages of abnormal nuclei were 19·09 and 13·45% respectively. These 2 groups differed significantly from control animals at the levels $P < 0·001$ and $P < 0·002$ respectively (Student's t test). The difference between the PCS and PCT groups was also significant ($P < 0·05$).

The proportion of glial nuclei to neurons was assessed in each specimen and no differences were found amongst the groups. There was no histological evidence of nuclear division in these sections. There was thus no evidence of any increase in the number or in the local turnover of glial cells as a result of the surgical procedures. No abnormalities of the neurons or their processes or of myelination were found.

DISCUSSION

The histological changes seen in the astrocytes of the dentate nuclei in this study are similar to those described in previous studies following portacaval shunting in rats (Doyle, 1967; Cavanagh and Kyu, 1969). While there was a small incidence of nuclear abnormalities in control and normal animals, there was a marked increase in such abnormal cells following both PCT and PCS. The incidence of these abnormal cells was, however, significantly greater after the latter operation.

It has been suggested that post-shunting encephalopathy is uncommon in the presence of normal liver function (Warren and Rebouças, 1964), and the clinical outcome of portal division procedures is better in patients with good preoperative liver function (Basu *et al.*, 1969; Sokhi *et al.*, 1975; Hourigan *et al.*, 1971). It has recently been reported, however, that long-term follow-up of children subjected to portal–systemic shunting in the presence of normal liver function has revealed the delayed onset of neuropsychiatric disturbances (Voorhees *et al.*, 1973). Similar results have been found in the late follow-up of patients who have undergone portacaval shunting for schistosomiasis (Goffi *et al.*, 1968). It is nevertheless possible that long-term deterioration may be related to subclinical liver damage induced by prolonged deprivation of portal venous blood. The greatest incidence of portal–systemic encephalopathy occurs after end-to-side portacaval shunting and this should probably be avoided in patients with significant hepatopetal portal blood flow.

It has recently been suggested that the portal venous content of endogenous insulin may be of importance in the maintenance of liver mass and function, and that diversion of this hormone is responsible for the atrophy which follows

portacaval shunting (Starzl, Porter and Putnam, 1975; Starzl *et al.*, 1976). Sustained high levels of insulin in peripheral blood following portacaval shunting may result in excessive removal of branched-chain amino acids by muscle with resultant excessive uptake of tryptophan by the brain (Munro, Fernstrom and Wurtman, 1975). This is supported by the finding of increased levels of serotonin in the brains of animals in coma due to experimental PCS (Baldessarini and Fischer, 1973), though normal levels may have been found in rats without coma after PCS (Doyle, 1967). The physiological role of the protoplasmic astrocytes may include neuronal substrate transport or the inactivation of synaptic transmitters, and impairment of these functions may be related to the metabolic changes which have been observed in association with hepatic encephalopathy.

PCT has been shown to produce portal–systemic shunting with maintenance of total hepatic perfusion at least equal to that of the normal animal (Heer *et al.*, 1963; Starzl *et al.*, 1962; Kreutzer and Schenk, 1971). Studies in our own laboratory have shown that total hepatic bloodflow remains unchanged up to 3 weeks after PCT in the rat (Ryan *et al.*, 1978). This operation results in a lesser degree of liver atrophy than end-to-side PCS and also in less marked deterioration in serum protein levels and liver enzymes (Benjamin *et al.*, 1976). A degree of liver atrophy and dysfunction does, however, occur, possibly related to deprivation of insulin or other "hepatotrophic factors", and a model of portal–systemic shunting with entirely normal liver function has not yet been achieved.

The present studies have shown a significantly smaller percentage of abnormal astrocytic nuclei following PCT than following PCS. These findings support the view that central nervous system damage is dependent upon an interaction between portal–systemic shunting and a degree of hepatocellular dysfunction. This liver dysfunction may be of a more subtle nature than we have been able to detect in our model at the present time, and further ultrastructural and biochemical studies in liver and brain using this model may help to elucidate the problem further.

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Increased Serum IgA Levels in Rats after Portacaval Shunt but not after Portacaval Transposition

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Groups of rats were submitted to end-to-side portacaval shunt, portacaval transposition or a control sham operation. There was an 18-fold increase of the IgA level in the serum of portacavally shunted rats ($n=7$) at 3–6 weeks after surgery. Gel filtration revealed that this increase was predominantly due to dimeric and polymeric IgA. The serum IgM of these shunted rats, but not the IgG, was also significantly increased (twofold) above their control values. In rats subjected to portacaval transposition ($n=6$), there was no significant increase of serum IgA, IgM or IgG when compared with their control values. These data confirm the active transfer, by the healthy rat liver, of IgA and, to a lesser extent, of IgM from the blood into bile. Impairment of this function, leading to the accumulation of polymeric IgA and IgM in the serum, in portacavally shunted but not portacavally transposed animals, may be related to impairment of hepatic blood flow and hepatic atrophy, shown earlier to occur in portacavally shunted but not transposed rats. Such reduction in liver cell mass could also explain the fall in transferrin levels in shunted rats.

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The concentration of IgA in rat bile is much higher than that found in rat serum [20]. This appears to be related to the capacity of the rat liver to clear polymeric IgA from the circulation into bile [7, 13, 19, 23, 30]. Such a mechanism provides an explanation for the rapid and selective increase of the level of IgA in serum after ligature of the rat bile duct [21]. In addition, procedures that reduce the number of healthy rat hepatocytes, such as subtotal hepatectomy and acute carbon tetrachloride poisoning, also lead to a large, but transient, rise in the serum IgA levels [14].

To determine whether diversion of portal blood away from the liver, as a result of natural or surgically induced shunting, has any effect on the metabolism of the polymeric immunoglobulins IgA and IgM, we have measured the serum concentrations of these proteins and IgG in normal rats and rats subjected to surgically induced end-to-side portacaval anastomosis (PCS). This procedure is, however, followed by a significant reduction in liver mass [2], and therefore, to determine whether any changes in immunoglobulin levels might be the direct result of portal diversion or merely the result of reduced liver cell mass, additional animals were subjected to portacaval transposition (PCT), a procedure that successfully results in diversion of portal blood away from the liver but does not result in a significant change in liver cell mass [2, 10, 24].

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MATERIAL AND METHODS

Animals. Male Sprague–Dawley rats weighing 250–400 g were used. Two separate control groups were used for the comparison with PCS and PCT groups. Each control group consisted of five littermates of the experimental group; controls were kept under the same conditions as the latter.

Surgery. All the procedures described were done under clean but not sterile conditions, and no antibiotics were administered. The animals were subjected to either end-to-side PCS [18] ($n=7$), PCT [26] ($n=6$), or a sham operation in which the liver was mobilized and the vessels displayed as in the first two groups (PCS and PCT).

With the animals under ether anaesthesia, blood was withdrawn pre-operatively and at weekly intervals post-operatively by puncture of the retro-orbital venous plexus. Serum was separated and stored at −20°C until assayed.

Immunochemical quantitation. IgA, IgM, IgG, albumin and transferrin were quantitated by radial immunodiffusion [22], using specific antisera [20, 21]. Rat serum albumin, purified by preparative electrophoresis [28], was titrated by the biuret reaction. Polymeric monoclonal rat IgA was purified from serum of rats carrying the IR-699 immunocytoma (courtesy of Dr H. Bazin, IMEX, UCL, Brussels, Belgium) by two successive gel filtrations on Ultrogel AcA 22, preparative electrophoresis, and passage through insolubilized [5] rabbit anti-albumin antibodies to remove IgA–albumin complexes. A single precipitin line was obtained at 8 g/l with anti-whole-rat-serum antibodies. IgM was similarly purified from the serum of rats with the IR-202 immunocytoma, kindly provided by Dr H. Bazin. IgG was eluted from DEAE-Sephacell with 0.05 M Tris–HCl buffer, pH 8.0. The concentration of the purified Igs was measured by absorbance at 280 nm, using an extinction coefficient ($E^{1\%,\ 1cm}_{280\ nm}$) of 13.5. For IgG, our anti-IgG antiserum revealed at least three precipitin lines corresponding to different rat IgG subclasses.

Since absorption measures the total IgG content of the standard, and radial immunodiffusion only measures its major subclass (major outer precipitin ring), IgG levels are expressed as percentages of the standard. Transferrin was not purified: values are given as percentages of a standard serum.

Gel filtration. Seventeen ml of serum from control or shunted rats, pooled from 3 to 6 weeks after surgery, were filtered on a 5 × 90 cm column of Ultrogel AcA 22 (LKB) in 0.02 M Tris–HCl buffer, pH 8.0, containing 1‰ sodium azide and 2% NaCl. Each eluate was concentrated 10- (shunted) or 15-fold (controls) by vacuum ultrafiltration, to quantitate IgA along the elution profile by radial immunodiffusion. Since the standard IgA was a mixture of dimers and higher polymers, the values obtained for IgA monomers were also divided by a correcting factor of 2.5, in order not to overestimate the monomers [11].

Agarose gel electrophoresis, immunoelectrophoresis and immunodiffusion. These techniques followed our standard laboratory procedures. Anti-rat-secretory component antiserum has been described earlier [20, 21].

RESULTS

The sequential mean levels of IgA, IgM, IgG, albumin and transferrin in the serum of the group of rats with PCS are compared with those of their control sham-operated group in Fig. 1. IgG and albumin levels showed no significant difference between the control and shunted rats. IgA levels of the PCS group were markedly increased (15- to 23-fold) during the last 4 weeks after surgery, when compared with controls. IgM levels also showed a moderate (1.7- to 2.2-fold) significant increase in the PCS group during the same period. The levels of transferrin were roughly twofold lower in the PCS group than in controls at all times after surgery.

When a group of rats with PCT was compared with its control group, no significant differences were found for any of the proteins tested (Fig. 2).

The molecular size of the IgA found in the sera of the PCS rats was compared with that of the IgA from the control group by gel filtration of pooled sera from both groups through the same column of Ultrogel AcA 22 (Fig. 3). The protein elution profile, measured by absorbance at 280 nm, was very similar for both samples, the IgA distribution being bimodal. The precipitin rings given by the control monomeric IgA, eluting slightly ahead of IgG, were the largest, seemingly indicating a predominance of IgA monomers. However, if the level of IgA monomers is corrected by the factor 1/2.5, then IgA polymers do slightly predominate over monomers in the control rat serum. In the pooled serum of the PCS rats, IgA polymers clearly predominated over monomers, whether the corrected value of monomeric IgA was used or not. The polymer to monomer ratios, corrected and uncorrected, are listed in Table I. Immunoelectrophoreses on the gel filtration fractions of both control and PCS sera confirmed these findings (not shown). Only traces of secretory IgA were identified, by immunodiffusion against anti-rat-secretory-component antiserum in the concentrated polymeric IgA fractions of both the PCS and control sera (not shown).

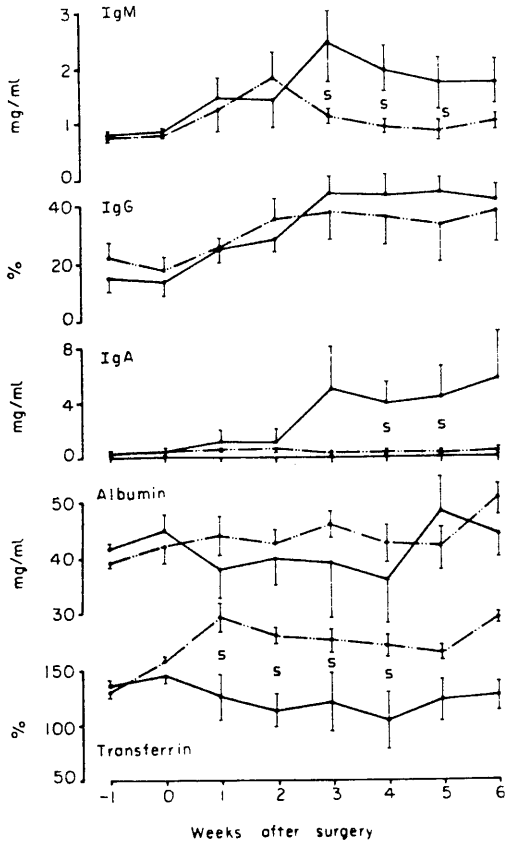

FIG. 1. Levels (mean ± SD) of IgM, IgG, IgA, albumin and transferrin in successive samples of serum from rats submitted to end-to-side portacaval anastomosis (●——●) ($n=7$) or to sham operation (●——··——●) ($n=5$), expressed in mg/ml for IgM, IgA and albumin, and in percentage of a standard (see Materials and Methods) for IgG and transferrin. S indicates a statistically significant difference between the means (Student's t test: $P<0.05$).

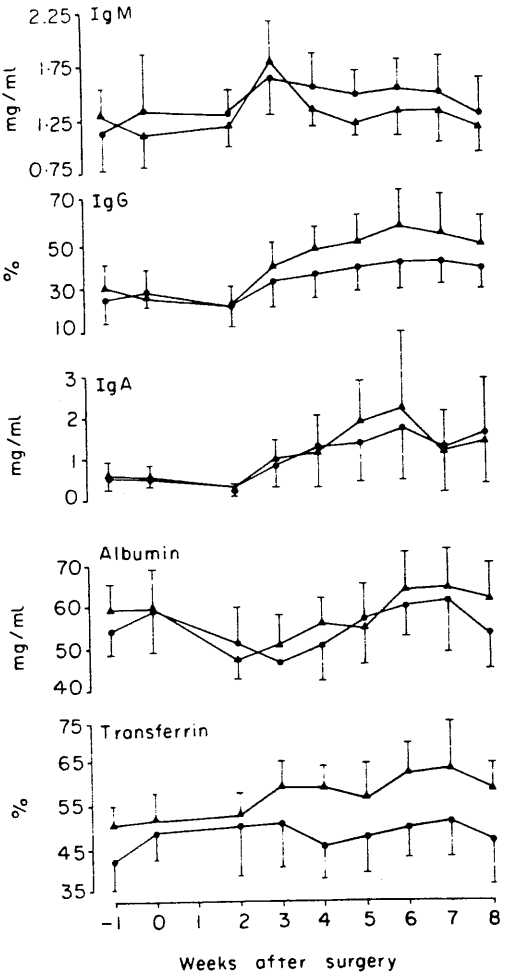

FIG. 2. Levels (mean ± SD) of IgM, IgG, IgA, albumin and transferrin in successive samples of serum from rats submitted to portacaval transposition ($n=6$) (●——●) or to sham operation ($n=5$) (▲——▲). There were no significant differences.

TABLE I. Ratios of polymeric to monomeric IgA levels* in sera of control rats and rats with end-to-side portacaval anastomosis

	Controls	Shunted
Uncorrected	0.78	3.05
Corrected†	1.94	7.26

* Levels measured by radial immunodiffusion of the gel filtration eluates (see Fig. 3).

† Correcting factor of 1/2.5 introduced to avoid overestimation of IgA monomers (see Materials and Methods).

DISCUSSION

The present data demonstrate that rats with PCS display large increases in their serum IgA levels, as compared with sham-operated controls or with rats with PCT. IgM levels in PCS rats were also significantly increased, albeit to a lesser degree. The IgA increase was predominantly due to polymers, monomers being much less affected. This increase of the polymeric IgA is consistent with a reduced capacity of the liver to transfer polymeric IgA from the portal blood

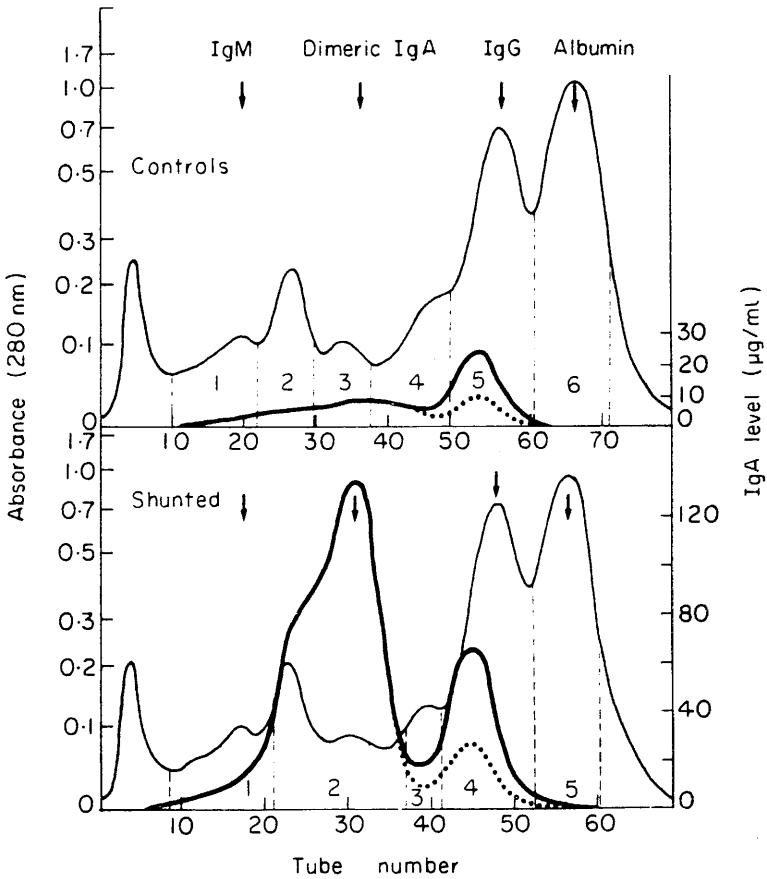

FIG. 3. Gel filtration on Ultrogel AcA 22 of serum pooled from 3 to 5 weeks after surgery from control rats or rats submitted to end-to-side portacaval anastomosis. Elution profile of total proteins (———) and distribution of IgA (—— or .....) along successive eluates. The corrected concentration of IgA monomers (see Materials and Methods) is illustrated by the dotted line.

into bile. The serum IgM increase could also be due to a reduced transfer into bile [7]. Serum IgG levels approximately doubled in PCS and PCT rats and in their respective controls, thus never reaching significance, in contrast to previous reports of significant increases in serum IgG after PCS [2, 16]. In one report, gammaglobulins were measured electrophoretically [16], a technique that does not accurately reflect IgG levels. In the second study, IgG was measured by radial immunodiffusion, using monospecific antiserum, and it remains possible that the specificity of this antiserum was different from ours in the recognition of the different IgG subclasses. This problem requires further study. It is noteworthy, however, that the spontaneous PCS developing in patients with extrahepatic portal vein obstruction does not result in increased IgG concentrations [32]. Even if the observed IgG elevations were due to surgery and/or infection, the fact remains that only IgM and IgA significantly increased above their littermate control values. Moreover, the insignificant IgA increases in the PCT group and its control were of a much smaller magnitude (Fig. 2) than in the PCS group (Fig. 1).

The increase in serum IgM and IgA levels was not apparent until 2–3 weeks after the induction of the PCS. This delay suggests that the increase is probably not directly related to diversion of portal blood away from the hepatocyte transport system but is a consequence of a reduction in liver cell mass occurring as a result of a decrease in total hepatic blood

flow [2]. Such a decrease in hepatocyte mass is known to take 2–3 weeks to develop [2] and is presumably the cause of the fall in serum albumin and transferrin in these rats.

The rat liver cell mass determines the efficiency of the IgA transport: partial hepatectomy and acute CCl_4 poisoning are followed by an increase in serum IgA levels by a factor of 10 within 2 to 4 days [14]. Hepatectomy only affects the number of hepatocytes, whereas CCl_4 could both reduce the number of hepatocytes and affect the function of the surviving cells. A metabolic hepatitis, induced in rats by galactosamine [15], also increases serum IgA levels within a few days (unpublished results). The failure of PCT to influence serum IgA concentrations further supports the concept that changes in functional liver cell mass, rather than portal blood diversion, is the major factor in determining the efficiency of the hepatic transport of IgA into bile. This transport occurs through hepatocytes, as shown by the autoradiographic demonstration of radiolabelled IgA in hepatocytes and bile canaliculi of rats injected intravenously with this protein [3, 4, 25, 31]. Whereas biliary obstruction resulted in a large reflux of secretory IgA into the serum [21], PCS produces an increase in serum polymeric IgA devoid of secretory component.

In humans, transport of polymeric IgA, locally synthetized in portal spaces, through bile duct epithelial cells into bile has been well documented [4]. Whether a hepatocyte transfer process also exists in man is still unknown. In patients with PCS after extrahepatic portal vein obstruction, total serum IgA levels were normal [32]. However, in cirrhotics the increase in serum IgA is deemed to be largely due to IgA polymers [1, 27], which are only poorly represented ($< 10\%$) in normal human serum [6, 28]. We are currently investigating the existence of such a system in man. Similar hepatic IgA transfer also exists in mice [12], rabbits [29,31], and probably in birds, since chicken, turkey and pigeon bile contain much more IgA than their respective sera [8, 9, 17].

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Portacaval Transposition in the Rat: Definition of a Valuable Model for Hepatic Research

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Portacaval transposition (PCT) in rats results in a smaller loss of body mass and liver mass than end-to-side portacaval shunt (PCS). Detailed studies of liver function, mass and histology were not previously available and have been undertaken in two different strains of growing rat in order to define the value of this model. PCT rats gained weight normally, while only 50% of PCS rats regained their preoperative weight by the tenth week. Wet and dry weights of liver fell relative to control values after both operations, but the fall was significantly greater after PCS than after PCT: there were parallel changes in hepatocyte size. There was a marked rise in liver-associated enzymes in the first 2 days after PCS only, and minimal enzyme elevations persisted in this group. The extent of cellular damage seen histologically closely parallelled the rise in SGOT in individual rats. At 72 hr, PCS rats showed focal necrotic changes, and by 10 weeks there was marked fatty infiltration: PCT rats had normal histology or showed minimal changes.

PCT therefore provides a model in which there is total portal diversion without the more severe effects of the conventional PCS on hepatic structure and function. This has particular value in studies of experimental hepatic encephalopathy, of hormonal and amino acid changes after portal diversion, and of factors initiating or controlling liver regeneration.

End-to-side portacaval shunting (PCS) in the rat (1), has been widely used as an experimental preparation. While this model achieves total portal-systemic diversion, associated progressive changes in liver mass and function make interpretation of results difficult. The operation of portacaval transposition (PCT) diverts the entire portal blood flow into the systemic circulation, but replaces the inflow to the hepatic portal tracts with systemic blood from the inferior vena cava. PCT was introduced by Child and his colleagues in 1953 (2). We have previously described the technique of PCT in the rat (3) and have confirmed that the procedure maintains normal total hepatic perfusion (4). Although the model has been used extensively in our laboratories (5-7), the basic characteristics of the preparation have not been formally defined, as has been done for the PCS (8, 9). This paper compares these characteristics with those of the PCS, and presents the evidence for relative maintenance of hepatic structural and functional integrity, upon which the value of the model rests.

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MATERIALS AND METHODS

ANIMALS

These studies were performed consecutively in two centers and in two different strains of rat: Sprague-Dawley (Glasgow) and Long-Evans rats (Cape Town). Animals were in the weight range of 290 to 390 gm at the time of operation. Housing conditions in the two centers were comparable, with an environment of controlled temperature and humidity and 12-hr cycles of light and dark. Four to six rats were housed in a cage, and there was free access to water and food. The laboratory diets were of equivalent composition (diet 41B, Herbert Styles Ltd, Budleigh, Yorkshire, England; Epol Rat pellets, Vereeniging Consolidated Mills, Maitland, Cape). During long-term experiments, all animals were handled and weighed weekly.

OPERATIONS

The operative techniques of PCS (1) and PCT (3) have been described previously. Control animals were subjected to laparotomy, mobilization of the vena cava and

portal vein, and clamping of these vessels for 15 min, a time comparable to that taken for PCT. Operations were performed under ether anesthesia, in clean but not sterile conditions, and no antibiotics were administered.

Experimental protocol

These experiments consisted of a long- and short-term study.

(a) *Long-Term Study.* Animals were weighed weekly and bled under ether anesthesia, and were sacrificed at 5 weeks (Glasgow study) or at 10 to 12 weeks (Cape Town study). At the time of sacrifice, final blood samples were withdrawn, a laparotomy was performed and the anastomoses inspected for patency. The livers were removed and weighed, biopsies cut from the median lobe and the remnant dried to constant weight in a drying oven and reweighed. A few animals with marked intraabdominal adhesions were excluded from the study.

(b) *Short-Term Study.* Blood samples were obtained at 6, 12, 18, 24, 42, 48, 66 and 72 hr by bleeding two groups of rats at alternate time points, and rats sacrificed as in the long-term studies.

Laboratory Methods

SGOT, SGPT, creatine kinase, lactate dehydrogenase and alkaline phosphatase were measured in Glasgow using a Beckman Autoanalyser system. SGOT was measured in Cape Town by a standard manual enzymatic method on a Beckman spectrophotometer. Serum albumin, IgG and α_1-globulin were measured by radial immunodiffusion assay (5).

Liver biopsies were fixed and embedded in a routine manner, and examined and reported blindly by a single histopathologist (J. A. H. C.).

Statistics

Results of liver and body weights are generally expressed as means and standard errors. Because of small numbers, data for some biochemical tests are given as median and range. Statistical comparisons were made using Student's t test or the Mann Whitney U-test as appropriate. Values of $p < 0.05$ were accepted as significant.

RESULTS

One-hundred seventy-eight rats were finally available for study (Cape Town 116, Glasgow 62). Of these, 74 were studied in the short-term experiment (control 24, PCT 24, PCS 26) and 104 in the long-term experiment (control 43, PCT 31, PCS 30). When results are available from both strains of rat for comparison, no significant differences were found between the strains. Where appropriate, therefore, results from both groups of experiments have been pooled.

Survival and Behavior

Operative mortality following PCT is higher than that for PCS, but with practice is less than 10%.

None of the animals exhibited overt signs of encephalopathy, although the PCS rats were less active than the other groups and developed an ill-groomed appearance. PCT animals were indistinguishable in appearance and behavior from control rats.

Body Weight

The body weight changes in Long-Evans rats studied over a 10-week period are shown in Figure 1. Weight changes in the Sprague-Dawley rat have been previously reported (3) and were essentially the same. All the PCS rats lost weight (mean maximum weight loss 19.1% ± 6.5 S.D.), and only 4 of 8 had regained their preoperative weight by 10 weeks after surgery. PCT animals suffered a small, transient weight loss after surgery (mean maximum 4.5% ± 4.4 S.D.), and 7 of 8 had passed their preoperative weight by the second week after surgery.

Liver Weight (Table 1)

The PCS rats showed a significant fall in both wet and dry liver weight at 10 weeks when compared with control and PCT animals ($p < 0.001$). The wet and dry liver weights of the PCT animals were less than those of control rats, although this difference only achieved statistical significance for the dry liver weight.

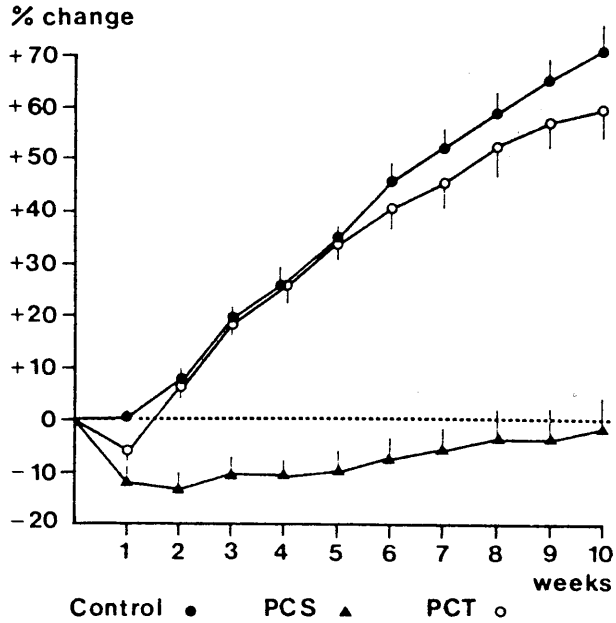

FIG. 1. Changes in body weight 1 to 10 weeks after surgery (Long-Evans rats). Each point represents percentage change from preoperative weight, as mean ± 1 $S.E._m$ in 10 to 16 rats.

TABLE 1. RELATIVE LIVER WEIGHT (PER CENT BODY WEIGHT) AT SACRIFICE 10 TO 12 WEEKS AFTER OPERATION (LONG-EVANS RATS; MEAN ± 1 $S.E._m$)

Relative liver weight	Control	PCT	PCS
Wet	3.05% ± 0.12	2.76% ± 0.10	2.00% ± 0.15[a,b]
Dry	1.10% ± 0.06	0.94% ± 0.04[c]	0.67% ± 0.13[a,b]

[a] Significantly different from PCT $p < 0.001$ (Student's t test).
[b] Significantly different from control $p < 0.001$.
[c] Significantly different from control $p < 0.05$.

LIGHT MICROSCOPY

In the short-term experiment, the only changes in the control and PCT groups at 72 hr were of mild to moderate fatty infiltration in the hepatocytes with fat deposits not distorting the nuclei in 3 of 11 control rats and 1 of 8 PCT rats. In the PCS group, however, 8 of the 11 animals showed central necrosis with infiltration of fibroblasts, lymphocytes and plasma cells. In the two animals most severely affected this was accompanied by fatty change and bridging necrosis. Only one liver appeared quite normal, and in two there was fatty change present centrally. Mitotic figures were numerous in some PCS animals.

At 10 to 12 weeks, only one animal in the control group and one in the PCT group showed a mild degree of fatty change of hepatocytes in the central and midzone. By contrast in 7 of 10 PCS rats, there was peripheral fatty change, graded in five as moderate to severe, usually distorting the nuclei and associated occasionally with necrotic cells.

As an index of hepatocyte atrophy in the long-term study, the degree of nuclear crowding was estimated, counting the number of nuclei in a high power field by means of a measuring graticule. Peripheral and central zones of the liver lobule were counted separately (Table 2). In both the control and PCT rats, the hepatocyte count was higher in the peripheral zone than in the central zone ($p < 0.05$). This difference was not apparent in the PCS rats, but these animals showed a very much higher hepatocyte count than the control and PCT groups ($p < 0.005$). This was particularly evident in the central zone where the nuclear count was almost twice that of control and PCT animals, confirming the visual impression of hepatocyte atrophy in these rats.

SERUM ENZYME CHANGES

(a) *Long-Term Study.* Figure 2 shows the mean percentage change in SGOT, SGPT and alkaline phosphatase in the Sprague-Dawley rats at weekly intervals until sacrifice at 5 weeks. At no time did enzyme levels in the PCT group differ from control values. While enzyme rises in the PCS group were generally modest, the SGOT level differed significantly from control at 2 and 3 weeks, and the SGPT level at 2, 3 and 5 weeks. The SGPT level was significantly higher after PCS than after PCT at all time points except the fourth week. Alkaline phosphatase levels were significantly different from controls from the second week onwards.

(b) *Short-Term Study.* Median values of SGOT from 6 hr to 7 days after surgery are shown in Figure 3. PCT rats reached a peak median level of 560 units per liter at 6 hr with a range of 427 to 2400. In the PCT group, there were only three values above 750 units per liter recorded in a total of 53 samples collected over the first 72 hr. In total there were only nine samples in this group with a value over 500 units per liter.

PCS animals showed a peak median SGOT level of 2,400 units per liter, at 12 hr after surgery (range, 520–7,245). Only six samples of 42 in the first 48 hr had values of 500 units per liter or less. Using the Mann-

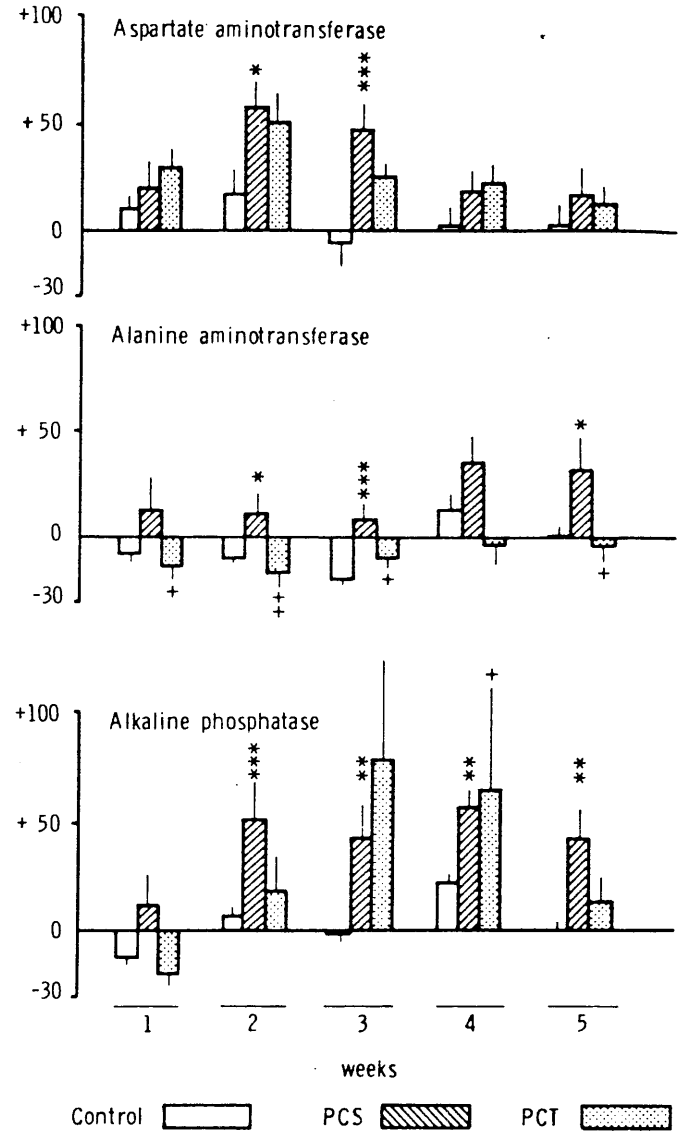

FIG. 2. Aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT) and alkaline phosphatase 1 to 5 weeks after sham operation, PCS and PCT in Sprague-Dawley rats. Each result is shown as percentage change from preoperative value ± 1 S.E.$_m$. Significance levels (Student's t test):

	PCS vs. Control	PCS vs. PCT
$p < 0.05$	*	+
$p < 0.01$	**	++
$p < 0.005$	***	

TABLE 2. HEPATOCYTE NUCLEI PER HIGH-POWER FIELD IN THE PERIPHERAL AND CENTRAL ZONES OF A LIVER LOBULE 10 TO 12 WEEKS AFTER SURGERY (MEAN ± 1 S.E.$_m$)

	Central		Peripheral
Control (n = 10)	26.5 ± 1.74	←p < 0.05→	31.1 ± 1.68
PCT (n = 8)	25.3 ± 2.37	←p < 0.05→	32.1 ± 2.90
PCS (n = 9)	49.7 ± 6.23[a]		50.4 ± 4.33[b]

[a] Significantly different from control and PCT, $p < 0.005$.
[b] Significantly different from control and PCT, $p < 0.001$.

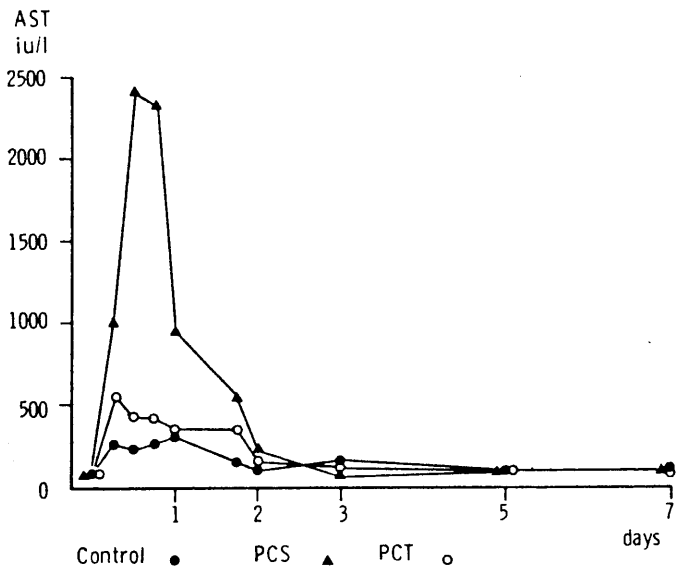

FIG. 3. Median values of plasma AST (SGOT) (IU per liter) 6 hr to 7 days after sham operation, PCS and PCT (n = 3 to 13 at each time point).

Whitney U-test, the PCS animals differed from controls at 12, 18, 24, 42 and 48 hr ($p < 0.05$). The PCT animals differed from controls at 12, 24, 42 and 48 hr, and from PCS animals at 12, 18 and 24 hr ($p < 0.05$).

SGPT, lactate dehydrogenase, creatine kinase and alkaline phosphatase were measured in a small study using three animals in each experimental group, with six hourly blood sampling (Sprague-Dawley rats). Changes in these enzymes were not as marked as those noted in the SGOT values during this period, but values of SGOT were consistently higher in the PCS group than in the control and PCT groups, which showed no significant rise. Values of lactate dehydrogenase were also higher after PCS, but no consistent changes in creatinine kinase and alkaline phosphatase were found.

SERUM PROTEINS

These changes have been reported in detail elsewhere (5). γ-globulin rose steadily and equally in both PCS and PCT groups during the 5-week period of study, and differed significantly from control from the second week onwards. Albumin levels fell in both the PCS and PCT group, and lower levels were seen after PCS than after PCT during the first 4 weeks. Slow α_1-globulin initially rose in all three groups, and these levels were maintained in the control and PCT rats, while the PCS group fell progressively from the second week onwards.

DISCUSSION

The general effects of PCS in the rat have been well described, and are confirmed in these experiments. The animals lose weight, apparently due to marked anorexia of undetermined cause (10). PCT rats, however, consume a normal diet, and in their growth, appearance and general behaviour are indistinguishable from control animals. Liver mass falls disproportionately to body weight in PCS animals, wet and dry relative liver masses being respectively 35% and 39% less than control animals ($p < 0.001$). There was a lesser, although significant, reduction in relative dry liver mass in the PCT group (14.5%), but values of both wet and dry weight remained significantly different from PCS ($p < 0.001$). Since total liver perfusion has been shown to be maintained at the preoperative level 5 weeks after PCT (4), this reduction of mass is presumably due to loss of hormone or nutrient input directly to the portal tracts of the liver, in keeping with the work of Starzl and his colleagues (11). Nevertheless, we have shown previously that despite this atrophy the hyperplastic (regenerative) response of the liver to partial hepatectomy is unimpaired after PCT (7).

Nuclear crowding in histological sections [a technique used previously by others for morphological assessment of the shunted liver (12)] confirmed the occurrence of hepatocyte atrophy (Table 2). It is also interesting that in the PCS animals, with no inflow to the portal venous radicles, the size distinction between hepatocytes in the peripheral and periportal zones of the liver lobules is lost. This suggests that if the hepatocytes of the periportal zone enjoy a metabolic "advantage", this is related to blood flow *per se* and does not depend entirely upon direct access to a high concentration of hormones or nutrients carried in portal venous blood.

In addition to this cellular atrophy, fatty infiltration of the hepatocytes is well established by 10 weeks after PCS, associated in some animals with cellular necrosis. Less marked changes were evident as early as 72 hr after PCS, with areas of central necrosis or bridging necrosis. This presumably reflects the abrupt reduction in sinusoidal flow which occurs in this group and is reflected also in the striking early release of intracellular hepatic enzymes following this procedure.

The most marked enzyme change after PCS was the gross elevation of SGOT, maximal at 18 hr and thereafter declining towards normal. PCT rats and control animals showed a modest change presumably associated with transient ischemia during portal occlusion. Marked rises in SGOT following PCT have in our experience been related to technical operative errors, resulting in hepatic necrosis and sometimes in postoperative death: no such animals have been included in these studies. After the first 7 days, differences in serum enzyme levels among the groups are smaller, but significant differences were nonetheless found between PCS and control rats, and between PCS and PCT rats.

The diminished levels of the liver-synthesized proteins albumin and slow α_1-globulin have already been reported in detail (5). These remain essentially normal in PCT rats, but are reduced from 2 to 5 weeks following PCS. Fisher and his colleagues (13) have demonstrated that dietary nitrogen absorption and retention are unimpaired following PCS when compared with pair-fed control rats. We have also observed normal nitrogen balance in PCT and PCS rats housed in metabolic cages (unpublished data). Even when pair-fed against PCS rats, PCT animals do not show the degree of liver atrophy seen after PCS (unpublished observations). Thus, the control of liver mass and hepatic protein synthesis in this model

appears to be related to the maintenance of hepatic tissue perfusion rather than to dietary factors.

Unlike dogs, rats do not exhibit spontaneous coma after PCS, although changes in motor activity (8), altered sleep patterns (14) and electroencephalographic abnormalities (15) have been shown. Although we have not quantitated these observations, PCT rats do not display the irritable behaviour on handling which is invariably seen after PCS. We have also previously demonstrated fewer histological changes in the central nervous system after PCT than after PCS (6), and have confirmed these findings in animals from the present series (Benjamin et al., data in preparation).

PCT provides a model of total portal diversion without the more severe effects of conventional PCS on hepatic structure and function. This preparation has been used to examine the phenomena of atrophy and regenerative hyperplasia following liver resection (7), and has proved of value in distinguishing between these co-existent but independent phenomena. Similarly, a comparison of PCT with PCS may allow elucidation of the complex interrelationship of hepatic dysfunction and portal diversion in hepatic encephalopathy. We feel that in these and other fields, the portacavally transposed rat offers a major extension to the repertoire of the experimental hepatologist.

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THE EFFECT OF PORTACAVAL SHUNTING AND PORTACAVAL TRANSPOSITION ON INTRASPLENIC OVARIAN AUTOGRAFTS IN THE RAT

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Running Title:

PORTAL DIVERSION AND INTRASPLENIC OVARIAN GRAFTS

INTRODUCTION

The changes in the ovary of the spayed female rat after implantation into the spleen were fully documented by Biskind and Biskind in 1944. Such autografts undergo a sequence of histological changes culminating in the formation of hyperplastic masses of luteal cells. The experiment has since been repeated in the rat (Peckham & Greene 1952 ; Furth and Sobel 1947 ; Gardner, 1955) and in other species. It is believed that this phenomenon is caused by excess production of gonadotrophins resulting from inactivation of ovarian hormones in the liver, and this is supported by the regression of the "tumours" after pituitary ablation (Kullander, 1956). It has recently beeen shown that such implants result in a rise in plasma FSH and LH concentrations. Portacaval anastomosis can prevent this rise, and can also block the growth of implants and produce atrophy of established implants (Seager et al 1974). Although this effect is probably due to the return of portal blood, and therefore ovarian hormones, into the systemic circulation, portacaval shunting causes liver atrophy and deterioration in liver function (Weinbren et al 1972), so that alteration in the hepatic metabolism of steroid hormones may be an additional factor. In addition, rats with portacaval anastomoses invariably lose weight, and their general well-being declines and atrophy of the implant may be merely a part of this more generalised deterioration.

Portacaval transposition diverts portal venous blood into the systemic circulation while maintaining normal total nutrient liver blood flow (Ryan et al 1977). Following portacaval transposition

in the rat body weight is better maintained, and liver atrophy is significantly less, than after end-to-side portacaval anastomosis (Ryan et al 1974). Liver function has been shown to be better maintained after portacaval transposition in the dog (Starzl et al 1962) and in the rat (Bejamin et al, 1976). Portacaval transposition has no effect on the regenerative capacity of the liver following partial hepatectomy (Guest et al, 1977). This paper reports an investigation into the effects of portacaval transposition on the growth and histological appearance of ovarian splenic autografts in the spayed rat, and a comparison with the effects of end-to-side portacaval anastomosis.

MATERIALS AND METHODS

Female Sprague-Dawley rats of 203-254 G. were used and were fed on rat cube diet 41B and water ad libitum. All animals undergoing implantation were in metoestrus or dioestrus, determined by vaginal cytology. Under ether anaesthesia bilateral oophorectomy and implantation of one half of the left ovary beneath the splenic capsule was performed. A flap of omentum was sutured over the graft in order to minimise adhesions (Seager et al 1974).

Twenty-six to 38 weeks after implantation the animals were re-anaesthetised and subjected to either laparotomy alone, end-to-side portacaval anastomosis (PCS) by the method of Lee and Fisher (1961) or portacaval transposition (PCT) by the method of Ryan et al (1974). At the time of

this second operation the graft was exposed, measured and photographed. The presence of adhesions was noted and when found these were ligated and divided. Most animals were sacrificed 20 weeks after the second operation, although 4 rats were sacrificed at earlier intervals. (See Table 2.) The implants were excised and fixed and embedded, and sections cut and stained with H and E for histological examination. In addition, several animals were sacrificed 26-28 weeks after implantation with no further operative procedure and the implants removed for histological examination.

RESULTS

The only mortalities in the experiment were due to an outbreak of pneumonia in the rat colony. Material from these animals is not included in the results described.

General Effects

Both PCS and PCT in the rats were well tolerated. As we have reported previously (Ryan et al 1974) both groups lost weight initially, but while the PCT rats soon regained their original growth curve, the PCS rats remained underweight for some time after surgery (Table 1).

Changes in Dimensions of the Grafts (Table 2)

There was a wide variation in the dimensions of grafts examined at the second operation. Solid tumour masses ranged from 5 to 15 mm. maximum diameter, and cysts of up to 30 mm. were seen in some animals. The relative prominence of solid or cystic elements was a variable feature.

Vascular adhesions in the laparotomy group make direct comparison with the PCT and PCS group impossible. All but one of the rats in the PCS group showed a decrease in graft dimensions. This rat had adhesions to the serosa of the stomach, which may have resulted in a degree of portal-systemic shunting before construction of the formal surgical shunt. 5 of the 7 rats in the PCT group showed an unequivocal decrease in graft size. The two rats which showed no clear-cut atrophy had a prominent cystic element in the graft making evaluation of changes in size difficult.

Histological Changes in the Grafts

The histological changes observed in ovarian autografts in other studies were confirmed in our control animals with no further operative interference following implantation, and in animals subjected to laparotomy alone 20 weeks after implantation. These appearances are shown in Figures 1(a) and 1(b). Luteal cells are seen massed into corpora lutea which may be so tightly packed as to compress and distort the remaining ovarian stroma into a thin capsule. These luteal masses often have fibrosed or cystic centres. At later stages the masses fuse and become less distinct, and develop a dense connective tissue stroma, without evidence of massive fibrosis or haemorrhage. Follicular cells, when present, are found lining cysts. These may be small, having the appearance of normal ovarian follicles, or very large, with a low epithelial lining and containing eosinophilic material.

All animals after PCS and PCT showed typical degenerative

changes in the graft, and these are illustrated in Figures 2 and 3. In one rat, regressive changes were shown as early as 2 weeks after PCS. The most prominent feature was degeneration and atrophy of luteal cells, whose presence could often only be inferred from the "ghosts" of the typical luteal cell masses seen in normal implants. Follicular cells were infrequent and often atrophic, and organised follicle formation was rare. Loss of follicles was not so prominent a feature as degeneration of luteal cells. The organisation of the implant was completely broken down and replaced to a large extent by a dense fibrous stroma, with occasional haemorrhages.

None of the implants showed histological evidence of malignant change and no distant metastasis or local extension was found.

DISCUSSION

The formation of follicles and masses of hyperplastic luteal cells in ovarian tissue implanted into the spleen of the spayed rat has been confirmed in the present experiments. The effects of portacaval shunting and portacaval transposition on these ovarian autografts is not readily amenable to quantitative analysis. We have been doubtful of the value of graft size alone as a reliable estimate of the effects of further surgery for several reasons. Dissection of the graft from its associated omental mass for purposes of

measurement may produce adhesions and affect the subsequent behaviour of the graft. The graft is also partially embedded in the splenic substance, and measurement is necessarily approximate. Finally, changes in the size of the cystic elements of a graft are not a valid measure of the extent of growth or atrophy in the substance of the graft itself. Despite these limitations diminution in the size of the implants could be demonstrated in most animals in both the PCS and the PCT group.

The histological appearance of the implants, however, gives a reliable index of graft behaviour. A comparable degree and type of atrophy of the hyperplastic grafts has been demonstrated in this way following both operations. Due to adhesions in control animals there is no satisfactory group for comparison following laparotomy alone, but laparotomy has previously been shown to have no effect on the subsequent behaviour of implants (Seager et al 1974).

Seager & her collegues (1974) demonstrated that after portacaval shunting, graft atrophy was associated with a return to normal of the elevated plasma levels of FSH and LH seen after implantation and spaying. Since liver mass and function are relatively well maintained after PCT, our results suggest that this is due to portal-systemic shunting of venous effluent from the implant, and that altered hepatic metabolic function or deterioration in the general health of the animal plays little, if any, part in the process.

Early accounts of this model described the formation of true luteomata and granulosa-cell tumours (Biskind & Biskind 1944, Peckham and Greene 1952) and hepatic metastases have been produced after passage of such growths through several generations of mice (Furth & Sobel, 1947). No evidence of malignancy was seen in the present study, or in other recent studies, and it seems improbable that the growth of such autografts represents a step in true tumour formation. The present experiments further support the view that this phenomenon is due to a break in the inhibitory feedback control of gonadotrophin release by ovarian hormones, and that regression of the grafts after portal-systemic diversion is the result of restoration of this control mechanism.

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SUMMARY

Ovaries autotransplanted into the spleens of spayed rats form hyperplastic luteal cell masses. End-to-side portacaval anastomosis and portacaval transposition cause a comparable degree of atrophy and histological degeneration of these implants. The changes may be accounted for by alterations in the ovarian-pituitary hormonal control mechanism.

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FIGURE LEGENDS

FIGURE 1 (a) Rat No. 34. Ovarian tissue 46 weeks after implantation, showing massed hypertrophic corpora lutea and follicle formation. H & E.

FIGURE 1 (b) Rat No. 51. Ovarian tissue 38 weeks after implantation, showing corpora lutea separated by fibrous strands, with modelling of the adjacent contours. H & E.

FIGURE 2 (a) Rat No. 23. 46 weeks old implant, 20 weeks after PCT. Degenerate corpora lutea with typical appearances of "corpora lutea ghosts". H & E.

FIGURE 2 (b) Rat No. 13. 46 weeks old implant, 20 weeks after PCT. Cellular degeneration in corpora lutea. H & E.

FIGURE 3 (a) Rat No. 31. 46 weeks old implant, 20 weeks after PCS. Degeneration and "corpora lutea ghost" appearance.
(cf. Figure 2 (a)). H & E.

FIGURE 3 (b) Rat No. 16. 46 weeks old implant, 20 weeks after PCS. Cellular degeneration in luteal cells. (cf. Figure 2 (b)). H & E.

TABLE 1

CHANGE IN BODY WEIGHT AFTER OVARIAN IMPLANT AND RE-OPERATION

	No. of Rats	Mean Body Wt. at Implant (G)	Mean Body Wt. at 2nd Op. (26-38 wks) (G)	Mean Body Wt. at Sacrifice (44-58 wks) (G)
Laparotomy	4	233	338	356
PCS	10	229	372	274
PCT	7	234	364	363

PCS = Portacaval shunt PCT = Portacaval transposition

P (PCS vs PCT at sacrifice) $<$ 0.0005 by Student's t-test

TABLE 2

EFFECT OF PORTACAVAL SHUNT (PCS), PORTACAVAL TRANSPOSITION (PCT) OR LAPAROTOMY ON SIZE OF IMPLANTS

Second Operation	Time Second Operation to Sacrifice (wks)	Maximum Change in Size (mm)
Laparotomy	20	-
	20	-3
	20	*
	20	+6
PCS	20	-6
	20	-5
	20	-4
	20	+2
	25	-8
	20	-7
	20	-3
	10	-2
	18	-20
	2	*
PCT	20	0
	20	-6
	20	-5
	20	-1
	20	-10
	6	*
	20	0