PIG LIVER PERFUSION

A Role in Hepatic Assist?

THESIS

for the Degree of

MASTER OF SURGERY

submitted to the University of Cape Town by

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ERRATA:

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"The pig is a gay animal"

Sir Winston Churchill.

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Figure 2, giving details of the patterns of events following partial hepatectomy in the rat, was first presented by Dr R.H. Stead of the Department of Medicine at a meeting of the Liver Research Group, and has been included with his permission. This, together with numerous occasions upon which advice in biochemical and laboratory technique was rendered so willingly, is much appreciated.

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INTRODUCTION

Fulminant hepatic necrosis is one of the most dreaded complications in medical practice. Although rare, this syndrome carries a mortality of up to 90%, which has not been improved in the last 20 years of intensive effort. The principal reason for the employment of treatments of great complexity is the accepted significant capacity of the normal liver to regenerate. Hence any form of treatment serves to sustain the life of the patient in order that this regenerative capacity may have time to manifest itself.

This study relates to an investigation of one form of treatment of this syndrome - viz. the use of an animal liver perfused in isolation with the blood of the patient to provide an extra-corporeal hepatic assist. Since no form of "artificial liver machine" has yet been devised, this bizarre method constitutes an approach to fulfil liver function.

As an introduction to the study, the problems of the definition and pathogenesis of fulminant hepatic necrosis are explored. A precise definition is essential in order that a true evaluation of the mortality from the disease may be made. Attempts to define the pathogenesis of the neurological syndrome have centred around a defect in the metabolism of ammonia since clinical abnormalities of nitrogen metabolism have been noted, and to some extent, the syndrome is reproducible experimentally by the administration of ammonia. However, the precise pathogenesis is far from certain.

Considerable difficulty exists in the assessment of when to extend conservative treatment to include more radical measures. This emphasizes the necessity for a method of comparison, for which the staging of coma has been employed with partial success. The difficulties encountered in any form of comparative study are highlighted in a review of the forms of treatment available, with a resume of some world experience.

Since it appears that the only entity available at present to replace the liver in toto is another liver, the design of hepatic assist was explored. As a background to this, the methods of assessment of liver function have been elaborated to demonstrate the difficulties involved. Methods including total hepatectomy or the establishment of an ischaemic organ have provided some understanding of liver function but a great volume of work has resulted from isolated liver perfusion studies. Nonetheless, it is well recognised that these results cannot be compared with whole organ function in vivo. The methods chosen for use in this study include a wide spectrum of physical and biochemical characteristics selected from the work of a number of authors.

Since large animal liver perfusion constituted a new field of research at this University, the establishment of the technique is reported in detail, including selection of animals, pre-opera-

tive, anaesthetic and operative techniques, and especially the preparation and perfusion of the liver. A review of the literature resulted in the circuit design which was the first University of Cape Town model, to which various alterations have been made in the course of the development of the procedure over three years.

Initial experiments were designed to evaluate the response of the isolated liver to perfusion with homologous porcine blood and to assess the need for additions to improve function. Subsequently a study was performed to compare the effects of perfusing the pig liver with homologous porcine or heterologous human blood, in order to determine that the procedure could be offered with safety as a hepatic assist.

In the light of the physical and biochemical findings observed in this laboratory and in those of many other workers, the isolated pig liver was offered as a form of treatment to four patients with acute liver failure. The clinical courses of these patients are outlined in detail to provide evidence of the disease under review.

Although none of these patients survived, an encouraging improvement in the level of consciousness was noted. But a feature became apparent which had not been emphasised in any other work. This was the thrombocytopenia which constituted a hazard to the recovery of these patients. As a result of the severity of this complication, the clinical procedure was discontinued while a laboratory programme was undertaken to investigate the nature of, and solution to the problem.

The hypothesis was advanced that the decline in platelet levels could be related either to the trauma resulting from direct blood/gas contact in the oxygenator, or to the effect of the liver itself. In experiments designed to evaluate the contribution of the circuit in which perfusions were performed without livers, it was apparent that there was some decline of platelets related especially to the gas flows in the different oxygenators, but that this could not account for the whole problem. The incorporation of a pig liver into a perfusion using porcine blood did not result in further fall off in platelet counts, but a striking decline resulted from the use of pig liver and human blood. It was observed also that the platelet counts fell markedly within the first 30 minutes of perfusion, and that this was a direct effect of the liver. In a further evaluation of this phenomenon, the disappearance of platelets was noted to be accompanied by reduction in leucocyte counts and the levels of complement and antibody in human blood against pig cells.

Upon review of the literature, it was noted that the heterograft response to interspecies transplantation was accompanied by similar features and hence this was invoked as the cause. An attempt was made to prevent or reverse this response although little previous success had been reported. The attempt took two forms - (a) In homologous perfusions prevention of plate-

let aggregation to damaged endothelium as a result of poor preservation - i.e. an attempt to improve conditions of liver function and (b) in heterologous perfusions prevention of antigen-antibody complex-induced platelet aggregation by pharmacological means. Single experiments in a number of these directions all failed.

An alternative approach was to find a more compatible animal than the pig. The calf and the lamb were also shown to give results similar to those of the pig when the liver was perfused with human blood. However, the use of baboon livers from blood group compatible or incompatible animals showed a marked improvement. Thus at this time, the use of the baboon liver for hepatic assist would be advocated, with the proviso that in an attempt to prevent transmission of viruses from these animals to patients or personnel, a period of isolation should be completed before use of such animals for clinical purposes.

Thus the solution to the problem has taken a different direction from that originally envisaged. The problem of the heterograft response remains unsolved and poses a number of questions which lead to a wide field of research. In addition, considerable improvement needs to be made in the technique of liver perfusion as a means of preservation of the numerous functions of this complex organ.

CHAPTER ONE

FULMINANT HEPATIC NECROSIS

i) Definition

ii) Proposed pathogenesis - with emphasis on hyperammonaemia

iii) Principles of treatment - general review

iv) Staging of coma

v) Treatment - (a) conservative
(b) exchange transfusion
(c) cross circulation
(d) isolated liver perfusion

vi) Liver regeneration

vii) A model of hepatic coma

i) **Definition**

Fulminant hepatic necrosis is a rare but lethal complication of several forms of acute liver disease. It carries one of the highest mortality rates of medical diseases (287) and is becoming of increasing importance in view of the outbreaks of viral hepatitis in dialysis and transplant units (207). Diseases in which acute necrosis may become a complication include (i) hepatitis as a result of infection by a virus either of the short- or long-incubation type; (ii) the acute fatty liver of pregnancy, and (iii) more recently, drug-induced hepatitis, with especial reference to halothane (264). When hepatic coma supervenes in the course of such acute liver failure it should be regarded as a most ominous sign and is usually followed by death (19).

Only since 1950 has there been an increased interest in hepatic coma on the basis of acute liver failure, perhaps because more elaborate methods have been advanced since then to attempt to halt the course of the disease. The particular biochemical and clinical syndrome must be differentiated quite clearly from the derangement accompanying chronic progressive dysfunction of the liver as exemplified by the cirrhotic process. A marked difference is oberved in the mortality rates of the two forms of coma - 20% of patients die in chronic encephalopathy as for example following porta-caval shunts, whereas 80-90% of patients in acute hepatic coma succumb (49). In addition, the presentation of the syndrome is different from the experimentally induced anhepatic state.

The disease under review has recently been described as the acute onset of progressive jaundice with decrease in size of the liver, foetor hepaticus and hepatic coma. Biochemical associations are a decrease in the prothrombin index, and an increase in aspartate transaminase, bilirubin, blood ammonia and α amino nitrogen. As a fulminant illness, hepatic failure usually results within eight weeks of the onset of the disease (262).

ii) **Proposed pathogenesis**

At this time, the protean nature of the metabolic disturbances (91) consequent upon total massive destruction of the liver are only slowly being evaluated. This is not "a discrete, aetiologically homogeneous disease (51)". The nature of the response of the liver cell to various forms of assault is not known and hence the biochemical results are poorly understood. Probably there are many results of liver destruction which could lead to coma - in contradistinction to the anhepatic state, since there is absence of liver function plus tissue damage. The grim prognosis of the disease is due both to the presence of a host of intermediary metabolic defects which cause deficient energy metabolism, acid base imbalance and disordered clotting mechanisms, and also to complex associated disorders such as renal failure, gastro-intestinal haemorrhage, sepsis and high output cardiac failure (50).

For many years, a defect in ammonia metabolism has been incriminated as the cause of

hepatic coma, and there is considerable evidence to suggest that this may be the case (87). Although direct infusion of ammonia solutions of different concentrations and under different buffered conditions resulted in coma in dogs (67), there was no relationship between venous blood ammonia levels and the production of coma, and the concentrations required to produce coma were much higher than are usually observed clinically. The mechanism of ammonia toxicity has been postulated to be interference with the cerebral metabolism of ammonia by glutamine synthesis and reductive amination of α oxo-glutarate (243) (see Figure 1).

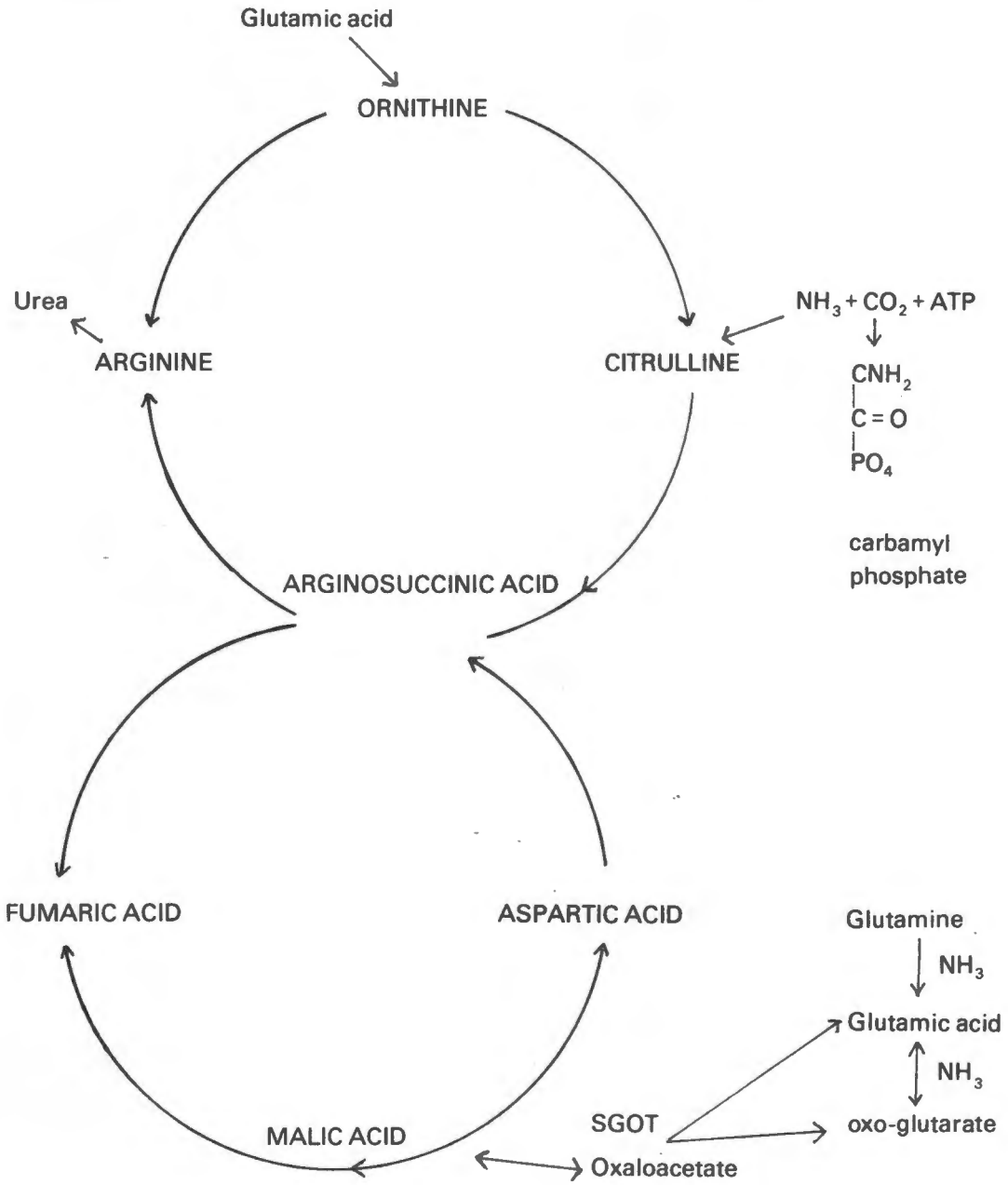

Figure 1: The biosynthetic pathways of ammonia

It is suggested that α oxo-glutarate is removed from the Krebs cycle with resultant decreased formation of other members of the cycle and diminution of oxidative phosphorylation in the brain (243). However, all these postulates relate the final result - viz, the elevation of blood ammonia, to the development of coma. Even if this were proven, the original cause of the elevation of blood ammonia is not known, although it is postulated to be a failure of the liver to metabolise the nitrogenous products of digestion. Studies of the isolated perfused pig liver have shown that the ability of the organ to excrete ammonia is indeed resistant to 30 minutes normothermic anoxia (275) and it is supposed that a great number of functions would have failed by the time that blood ammonia elevation was noted. Gabuzda (87) states - "Qualified acceptance of the role of ammonia in the pathogenesis of hepatic coma is related to such factors as the limitations of methodology, apparent lack of consistent correlation of blood ammonia levels with clinical state, and variations in the degree of elevation associated with the clinical syndrome from patient to patient, and in a given patient from time to time". Thus the problem is complicated by the tendency to regard hepatic coma and failure as synonymous with ammonia intoxication. Other factors which have been incriminated with less certainty include changes in the acid base status (110), and cerebral blood flow with disordered neurological transmission (204,80).

iii) **The principles of treatment - general review**

Since there are few, if any, metabolic functions in which the liver does not participate (223), replacement during its failure is complex, and various approaches have been made. Many authors advocate no more than the basic management of coma (19) but others follow the principles as outlined by Williams (287) - elimination or neutralisation of the toxic compounds thought to be implicated in cerebral toxicity; maintenance of fluid, electrolyte and acid base balance; the provision of sufficient caloric intake in the form of glucose infusions; the supply of the essential metabolites assumed to be lacking in the condition, and the control of the increased bleeding tendency. In a recent extensive review, Hoenig (121) concludes that "most forms of liver damage treatment are derived from clinical experience, personal impressions, experiments on animals and biochemical considerations rather than from well controlled clinical work. That is why polypragmasia has alternated with therapeutic nihilism".

Indeed, since the biochemical dysfunction is obscure, the basis of treatment rests on sustaining the life of the patient to provide time and the best conditions for the enormous capacity of the liver for regeneration to become effective. It is becoming increasingly apparent that the commencement of liver regeneration is the governing factor to the success of any form of treatment. Stated succinctly - "provided regeneration occurs, the condition is potentially reversible" (212).

However, in spite of increasing biochemical knowledge, the subject of acute liver necrosis and the high mortality rate associated with the condition remain a therapeutic challenge (150). Prior to 1951, it appears that intravenous infusion of glucose was the only form of treatment offered. Thereafter, with the advent of corticosteroids, these drugs were given in large doses, and for a period of time enjoyed popularity and apparent success in the treatment of some patients (133). However, their effectiveness was limited and their use empirical. More importantly, the complications of gastro-intestinal haemorrhage in patients with hepatic-induced coagulation defects, and enhanced tendency to infection were recognised.

Other forms of treatment of chronic encephalopathy with biochemical rationale included the use of sodium glutamate (291), a combination of threonine, nicotinamide and riboflavin (150), α oxoglutarate and ornithine (202), and ornithine and aspartic acid which have recently been proposed to spare the use of ATP in glucose metabolism (96). In addition, Levo-dopa has been advocated to improve the presumed disorder of neurological transmission (80,204).

With the realisation of the diverse complications of liver failure some attempts were made to provide an artificial organ (151). In broad summary, liver failure represents a deficit of excretion and a failure of synthesis, and whilst dialysis and exchange transfusion could supplement the former, another liver is needed for the latter (288). The object of further treatment was the same - viz. to provide temporary hepatic support of vital functions whilst regeneration occurred. But with the afore-mentioned difficulty in assessment of the cause of coma, and the nature of liver dysfunction, it has become apparent that the only means of replacing total liver function may be with another liver; nonetheless, on the basis that the coma might be due to accumulated toxins which could also hamper regeneration, the use of exchange transfusion was suggested (233,261).

The institution of any form of treatment other than conservative brings with it the necessity to select patients and the optimal time at which to employ such procedures. Because of the variable nature of the disease, and the absence of controls (212), and because no good objective standards exist to predict which patients will survive hepatic coma (153), the selection of patients becomes all important. As a result of the extremely high mortality rate associated with the disease, it is easy to develop a nihilistic attitude to such patients, especially as the main clinical indication of prognosis is the ill-defined conscious state (279). Thus in an attempt to provide guidance in the management of such patients, various gradings have been made of the stages of coma.

iv) **Staging of coma** (Table I)

This has been a contentious topic since the first definition by Adams and Foley made from their numerous observations of patients in chronic hepatic encephalopathy (10). This basis of

Table I : A comparison of assessments of hepatic coma

(a) Adams and Foley

(b) Trey

(c) Sherlock

(a) - Mental confusion with increased or decreased psychomotor activity
- stupor
- coma

(b)

Stage	Mental State	Tremor	EEG changes
1	Euphoria, occasionally depression. Fluctuant mild confusion. Slowness of mentation and affect Untidy. Slurred speech, Disordered rhythm.	slight	usually absent
11	Accentuated I. Drowsiness Inappropriate behaviour Maintenance of sphincter control.	present	Abnormal generalised slowing
111	Sleeps much of the time - rousable. Speech incoherent. Marked confusion.	present if patient can co-operate	always abnormal
1V	Not rousable. Variable response to noxious stimuli.	usually absent	always abnormal

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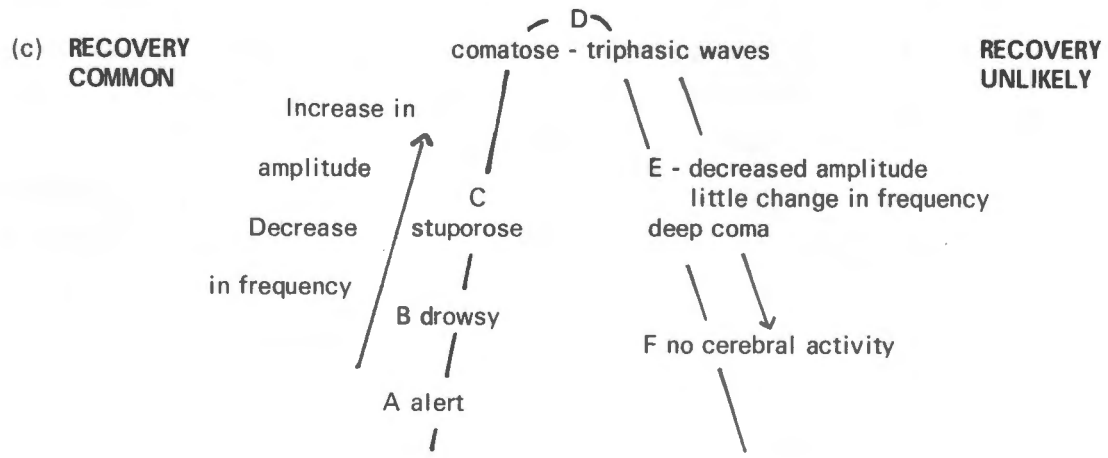
(c)



assessment was used for many years but more recently a classification directed towards the neurological disorders of acute liver necrosis has been evolved by Dr Charles Trey in the course of the Fulminant Hepatic Failure Surveillance Study. Subsequently in correlation with the electoencephalographic findings, six stages of coma were described by Sherlock (130). These various gradings may all be regarded as different stages in the evolution of understanding of the neurological deficits resultant upon acute hepatic necrosis, although it was recognised early that "the final stage is generally ushered in abruptly by grave nervous manifestations" (212).

v) **Treatment** (236)

(a) Conservative

At this juncture it is important to stress that intensive care may be the most important feature of the management of these cases. As the complications of hepatic coma are better understood, more success is achieved in the provision of intensive care. At present the following scheme is used in the Liver Unit, Groote Schuur Hospital.

Patients are nursed under isolated conditions and staff wear gowns, gloves and masks. Rigorous personal hygiene is insisted upon at all times and especially after any contact with the patient, blood or excreta; this has been highlighted since the recommendations of the Edinburgh group (207) following complications in their renal dialysis unit.

Complications to which these patients may succumb are legion. Hypoglycaemia is important and is treated by continuous infusion of dextrose with hourly monitoring with Dextrostix and frequent blood sugar analyses. In at least one patient, this metabolic defect has reached major proportions (232). It has recently been observed also that hypothermia may be a concomitant of the disease itself, in addition to the comatose state. In one patient, consciousness returned with rewarming after a fall in temperature. Respiratory failure has been recognised as a complication which may manifest suddenly, and hence tracheostomy is advocated as early as possible - often being performed immediately after an exchange transfusion when coagulation deficits are improved. In regard to this respiratory failure, certain drugs, principally Diazepam, seem to be implicated in its precipitation, and thus this form of sedation is not given. In a number of instances, death has seemed to be related to administration of paraldehyde. For both these drugs, the normal dose constitutes a major excess in the absence of liver metabolism. Patients are routinely catheterised to provide monitoring of renal function, and intake and output are carefully recorded. Antibiotics are administered only for obvious infection except for Neomycin which is given routinely to prevent protein intoxication, as is an initial Neomycin enema.

Many reports exist of various forms of treatment for hepatic failure and some of these are

summarised in Table II. The various results will be discussed in the description of each form of treatment. In only one of the series describing treatment by conservative measures is a more satisfactory survival rate shown (41). Perhaps a lesser severity of the disease in these patients could be inferred from the fact that liver biopsies could be performed early in the course of the disease, suggesting that coagulation defects at least, were not as profound as in other patients.

(b) Exchange transfusion

This was postulated as a form of treatment (261) which was safe and carried few hazards, and was thought to provide a means of removing whatever toxin it was which resulted in hepatic coma. As with all forms of treatment, there have been conflicting reports - some in favour of the method (23,261), and others showing no success with its use (130). Initially, the technique was extended from that used in exchange transfusion of jaundiced infants, and in general this method has been maintained. However, the routes used have been improved by the preparation of a Scribner shunt between the radial artery and an available forearm vein, which provides direct access to in and outflow. In only one report has the technique been rendered more complicated by the incorporation of a hand pump (186) - in this instance, direct exchange transfusion was performed from a succession of donors. This has been found unnecessary in local experience and carries the risk of exposure of an unnecessary number of people to the patient with a highly infectious disease.

In a recent further attempt to remove toxins, a carbon column has been used for the removal of bilirubin and alkaline phosphatase (18) and has been shown to clear drugs (227) but the problem of trauma to blood elements remains to be solved.

(c) Cross circulation

More active treatment has included the use of cross circulation techniques. For this purpose, human "volunteers" have been used in several series (37), but in view of the doubtful ethics of this method, a preferable technique has been the use of a non-human primate (125,235). Preparation of the animal included almost complete replacement of the blood volume with human blood compatible with that of the patient, whilst the animal was sustained on hypothermic cardio-pulmonary bypass. Subsequent to rewarming of the animal, its circulation was connected to that of a patient via Scribner shunts. In this one instance, it was found necessary to incorporate a pump between the patient and the baboon due to the discrepancy in size and the danger of overload of the animal by the human circulation.

The animal prepared in this way has been shown to survive normally for five days before any immunological response is noted (134), and this form of treatment has the major advantage of attachment of the patient's circulation to a liver under the normal homeostatic mechanisms of the animal. However, the virological hazard of the baboon has precluded the

Table II : Some world experience in the management of hepatic necrosis

Cons.	=	conservative
XT	=	exchange transfusion
PLP	=	pig liver perfusion
CC	=	cross circulation
haemo.	=	haemodialysis
V	=	viral
H	=	halothane
S	=	serum
D	=	drug
chr.	=	chronic liver disease
preg.	=	pregnant

No:	Aetiology	Type of Treatment	Survival	Rec. consc	Ref:
		Conservative			
13	V		4	4	184
207	various		3	36	24
61	V,S,D,		20	20	41
29	V,D,		3	3	53
		Exchange Transfusion			
31	V		1	4	223
7	V,H,		2	3	24
4	V,H,				259
1	V		1	1	23
7	V,D,		0	2	130
44	- -		11	11	57
1	V		1	1	153
5	V		2	3	267
		Liver Perfusion			
1	chr		0	1	224
5	V,D,preg.		2	5	201
1	V		1	1	277
5	V,chr.		0	2	240
8	chr		0	8	71
		Combination etc.			
5	V & chr	PLP & cons.	1	1	278
5	V,H,	XT & CC	1	4	37
7	V,D,H,chr	XT & PLP	0	3	49
1	preg.	XT & haemod.	1	1	213
4	V	XT direct	1	3	186
1	chr.	XT & PLP	0	1	6
2	V	XT & PLP	0	1	210

further use of this method until adequate isolation facilities have become available.

(d) Liver perfusion

In 1958, the pig liver was evaluated in experiments which resulted in the development of hepatic assist procedures (70,238). Initially the liver was perfused in isolation and was found to be capable of clearance of ammonia. This fact stimulated investigation of the ability of the liver to detoxicate ammonia even when perfused with heterologous blood (155). The removal of lactate was also demonstrated. At the outset, and in the absence of any further assessments of function, this technique was offered to provide these functions in the absence of an artificial organ (71). However, this constitutes a major procedure of similar magnitude to the cross-circulation, and in view of its bizarre nature, has only been offered as a last resort to patients in the most terminal phases of the disease. Indeed it has been stated that "such a difficult and possibly dangerous procedure, if used at all, should be reserved for massive liver cell necrosis from which recovery cannot be expected" (261). This attitude has detracted from the potential success of treatment but results from the difficulties involved in justification of earlier use of such radical methods.

Liver perfusion enjoyed popularity as a form of treatment in the 1967/8 period, but has tended to fall into disrepute more recently because of the small success rate. In addition, because of the complexity of the procedure, little monitoring has been performed of function of the liver during hepatic assist and hence failure of the technique may have related to lack of function of the extra-corporeal organ. Livers from pig, (71,73,115,191,211,279,288), calf (26,49) and man (6,241) have been used, and the techniques have fallen into two broad categories - either with a simple or a more complex circuit. In the former, a pump has been used to deliver blood from the arterial line of the patient directly into the portal vein of the hepatic assist liver, and blood returns from the vena cava directly to the patient (71). In the more complex circuit, a system similar to that used for isolated perfusion is used. The reasons advanced for the use of the latter relate to the provision of an adequate blood supply to the hepatic assist liver in the case of a patient who is a child, or who is hypotensive (49,118).

There is a great discrepancy in the duration for which livers have been perfused - it is stated that such organs will function between 10 and 35 hours (288) but again the question of the evaluation of function becomes important. Eiseman has suggested that four hours may provide the optimal period of perfusion before the law of diminishing returns becomes operational (73).

One of the most dramatic reports recently has been that of Abouna who has demonstrated support of a patient for 72 days by intermittent use of hepatic assist and exchange transfusion (6). Whilst difficult to evaluate from the ethical point of view as has been highlighted from the

correspondence which followed the report (54), it was shown quite clearly that in that particular patient, coma was more easily reversed by liver perfusion than by exchange transfusion (6). However, in other patients coma has been reversed by exchange transfusion, and in one patient 7 such procedures were required (261). These different data are impossible to evaluate in view of the fact that to date, no single centre has sufficient experience to show an improvement of survival. This dilemma may only be solved by the development of a suitable laboratory model. At present, however, pig liver perfusion continues to be used in patients with terminal disease (152).

The table shows some of the world experience with the treatment of hepatic coma, mostly based on acute hepatic necrosis. The current status of fulminant hepatic necrosis at Groote Schuur Hospital and the Red Cross War Memorial Childrens Hospital is as follows:- in the period 1963 to 1971, 119 patients in grades III and IV coma were seen. Of these, 48 were adults and 71 were children. In 20 of each group exchange transfusion was performed; in adults, 4 pig liver perfusions and one cross circulation with a baboon were used as additional treatment. A total of 10 adults survived (21%) and 24 children (31%). The difficulty of drawing conclusions from these figures has been highlighted in the differences in management and the assessment of severity.

vi) **Liver Regeneration**

Although so little is known of the biochemical defects of hepatic coma, probably the most important factor in the recovery of these patients is the capacity of the liver to regenerate. Hence at this point, a short evaluation of the present knowledge of liver regeneration control is pertinent. Numerous studies exist in rats where reproducible models of 68% hepatectomy can be produced (35). Whilst the biochemical, morphological and ultrastructural results of partial hepatectomy are becoming clearer, the humoral control mechanisms are still elusive. The present state of knowledge in summary is represented in Figure 2. Observations of altered rates of enzyme synthesis have been made within the first hour of partial hepatectomy (229) with associated increase in the specific activity of Uridine triphosphate (36) and mitochondrial (99) and nuclear DNA (36). Subsequently a pattern of increase in nuclear DNA specific activity has been shown, which is accompanied at 18 hours post-operatively by appearance of a factor capable of fibroblastic stimulation. This factor is RNA-ase sensitive (92). Growth hormone and non-specific surgical stress have been shown to increase the rate of response to the regeneration factor after hepatectomy, but only if administration precedes the operation (174). Further experimental studies using para-biotic circulation of rats have demonstrated conclusively that the stimulus to regeneration is independent of the liver since total hepatectomy in one partner results in stimulation of growth in the other liver (92). However, functional liver mass may be necessary for the processing of such a factor.

Figure 2 : A composite diagram of the events currently known to follow partial hepatectomy in the rat.

(i) Increase in the specific activity of uridinetriphosphate.

(ii) Increase in ornithine decarboxylase activity.

(iii) Increase in mitochondrial DNA specific activity.

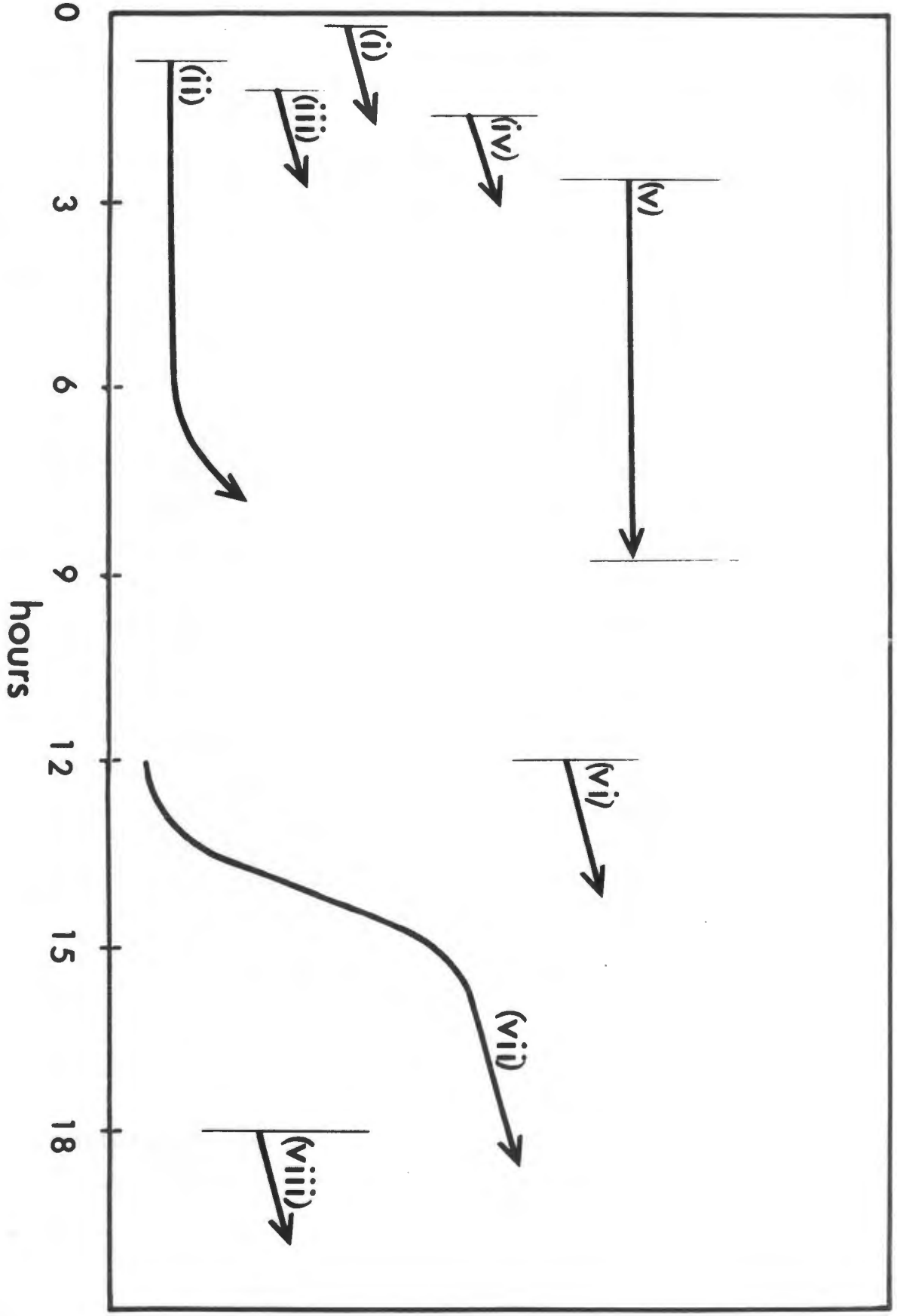
(iv) Increase in lipid.

(v) Disintegration of endoplasmic reticulum to form polyribosomes, with consequent reformation of endoplasmic reticulum.

(vi) Increase in nuclear histone.

(vii) Increase in nuclear DNA.

(viii) Increase in a factor which stimulates fibroblastic activity and inhibited by RNA-ase.


0
3
6
9
12
15
18
hours
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)

These studies emphasise the importance of liver regeneration in this disease and highlight at least two questions related to present treatment. One is whether livers damaged by massive necrosis in fact have lost the ability to respond to any regenerative stimulus, or whether if it could be isolated, administration of the regeneration factor might result in recovery of liver function. The second question concerns the validity of attaching such patients to whole livers, especially in cross circulation procedures. Indeed it might be proposed that the partners for such procedures should undergo partial hepatectomy to increase production of regeneration factor.

vii) **A model of hepatic coma**

As discussed in the section evaluating various forms of treatment, the availability of a model in which these could be tested is much needed. Numerous attempts have been made, but many involve the creation of an irreversible lesion by anatomical disturbances - as for example in the three stage operation of Rappaport (220) with ligation of the common hepatic artery with partial Eck fistula formation, followed by total ligation of all portal and hepatic arterial radicles. In addition chronic encephalopathy has been induced by the creation of an Eck fistula in dogs.

Whilst these studies provide interesting results as to the biochemical abnormalities of chronic liver failure, little contribution is made to acute necrosis. The large number of toxins which may induce experimental liver injury is reviewed by Schiff (108) but the only report of liver necrosis analogous to that seen clinically resulted from the use of galactosamine in rats (135). Thus, in general, little useful information in regard to the management of the clinical problem has come from the experimental induction of necrosis. The mouse and the dog are the only species in which viral hepatitis can be induced at will by infection, and in these cases, the disease is widespread and massive, involving many lobules. In addition, for practical purposes of biochemical evaluation, the more easily available mouse is too small.

A recent report by Trey holds more hope (263). A syndrome similar to that seen clinically was induced in monkeys by the direct injection of carbon tetrachloride into the mesenteric vessels. The technique resulted in fulminant hepatic necrosis analogous biochemically and clinically to that caused by the virus in humans, and initial reports showed that there was a statistically significantly improved survival rate in animals treated by exchange transfusion when compared with their matched controls. This confirmed previous work by Turcotte (267) using the anatomical technique of Rappaport, in which an improvement in the neurological status but not the survival rate was noted. Exchange transfusion of hepatic failure induced in the dog by the canine hepatitis virus resulted in a survival of three animals of thirty-nine, whilst none of the controls survived.

Initial studies of the value of the perfused liver were performed in dogs in which a porta-caval shunt had been created (200). The technique reversed the meat intoxication syndrome which was induced in these animals.

The use of the anhepatic animal for assessment of the value of cross circulation provides only a limited period of time before irreversible changes, perhaps haemodynamic, supervene (253), and attempts to treat such animals in the terminal phases of the experiment were unrewarding (132). This raises the question - has the patient with massive liver necrosis reached a terminal stage as seen in animals following hepatectomy, when reversal of the final syndrome has not been achieved by any form of treatment? The answer almost certainly lies in the capacity of the liver to regenerate.

CHAPTER TWO

THE ASSESSMENT OF LIVER FUNCTION

Introduction

i) Total hepatectomy

ii) Ischaemia (a) general
(b) the nature and process of biochemical dysfunction
(c) reversibility
(d) the duration - including the value of histology

iii) Methods chosen in this study

(a) Physical characteristics - resistance
total flow
pressures
bile flow rate

(b) Biochemical characteristics - cell membrane integrity
glucose metabolism
oxygen consumption and lactate/pyruvate ratio
acid base balance
parenchymal damage

(c) Biliary secretion

iv) Excretory characteristics and clearance

v) Synthetic ability

vi) Reimplantation

Introduction

Due to its metabolic complexity, the assessment of the function of the liver is extremely difficult. No single test represents total hepatic function and indeed it is thought that with different degrees and types of damage, different abnormalities manifest. One of the most important but indefinable aspects of the assessment of function is the question of the reversibility of any abnormal features which may be noted.

In attempts to evaluate the function of the isolated liver, at least three approaches may be cited which have contributed to assessment of the perfused liver. These are:- (i) the effects of total hepatectomy upon subsequent biochemical patterns; latterly this has been further extended during transplantation procedures when a poorly stored liver may be implanted. (ii) The assessment of the results of ischaemic liver necrosis and (iii) studies on the perfused liver itself - from both rats and larger animals.

i) **The effects of total hepatectomy**

This technique was first investigated from 1920 by Mann, Bollman and Magath in an attempt, by total removal of the organ, to analyse the resultant disorders and hence to derive its functional ability (164). Overall, these experiments showed that after total hepatectomy, an animal could be kept alive, awake and normal for several hours with the infusion of dextrose. Terminally, after some 18 to 36 hours, drowsiness and coma ensued, accompanied by a progressive metabolic acidosis which was not reversible by conventional methods. Muscular twitchings, hypotension, Cheyne Stokes respiration and diminished urine output were also noted. Addition of glucose reversed most of these features for a time, and other anti-hypoglycaemic agents including maltose, galactose, mannose, dextrin and glycogen were also effective. However, a number of other substances which relate to glucose metabolism and acidosis were found to be ineffective - these including saccharose, lactose, laevulose, insulin, sodium chloride, sodium sulphate, sodium bicarbonate and carbonic acid, ethyl alcohol, glycerol, lactic acid, acetic acid, hydrochloric acid, pyruvic acid, epinephrine, pituitary extract and glycoll (164).

In addition to the hypoglycaemia, there was a marked and progressive decline in urea levels, provided that the urine output was adequate, and a continued increase in a amino nitrogen. There was some excretion of uric acid and bilirubin into the urine but in general these substances accumulated in the blood. The animals also lost their ability to detoxicate various drugs.

A more recent evaluation by Drapanas et al (60) has confirmed these observations and made a few additions. The most important of these was that there was no further evidence of anything other than ammonia for the "toxic" factor responsible for death. They added several

observations to the biochemical changes already recognised - viz. - the decline in pH, increase in serum potassium and glutamine and in regard to cerebral metabolism, a decreased glucose and α oxoglutarate despite elevated serum levels of the latter.

At the stage when no further treatment was effective, the use of para-biotic circulation or isolated liver perfusion was shown to reverse the syndrome (253,144), thus suggesting that the only treatment able to reverse the initial defects resulting from an absent liver was the insertion of a liver into the blood circuit. It was confirmed that the liver is entirely responsible for protein, urea and glucose synthesis and that there is an integral role in the function of the clotting mechanism (164,154). This last important observation has only more recently become apparent. Attempts to store the liver with subsequent transplantation have demonstrated clearly that the first biosynthetic defect to manifest in a poorly stored organ is a bleeding tendency (205). Despite a considerable amount of investigation, the precise cause has not yet been defined. It is known that the liver is responsible for synthesis of most of the clotting factors (6,281). Also a "scavenging" role has been proposed as Kupffer cells in the liver clear the circulation of fibrinogen degradation products and hence prevent the development of disseminated intravascular coagulation (89).

(ii) **Ischaemia**

(a) Introduction

An approach has been made to investigate liver function by observing the effects of liver ischaemia. Again the question of the reversibility of changes arises. In addition, the effects of ischaemia are difficult to assess in vivo, although attempts have been made in the dog to prepare a model with isolated vasculature but still under the control of various hormonal mediators (95). In the pig, such a study is rendered more difficult by the short length of the hepatic veins which drain directly into the inferior vena cava and are inaccessible. Hence it cannot be assured that hepatic venous blood sampling is not contaminated with vena caval and thus renal effluent. Diversion of the vena caval blood into the jugular vein has been considered, but this could be hazardous in the unanaesthetised animal and variations in haemodynamics might occur. In addition, the flow of vena caval blood through the liver may be responsible for the removal of products of metabolism to the general circulation. Hence the majority of information on liver ischaemia has been accumulated from isolated perfusion experiments. Yet it has been suggested that such a perfused organ may have been subjected to hypoxia, hypercarbia and elevated levels of adrenaline and noradrenaline during hepatectomy (100), and that these effects could be carried over into the perfusion experiments.

(b) The nature and process of biochemical dysfunction

A number of workers have attempted to define precisely the initial abnormalities which

occur. Two phases of response have been suggested - (i) a response of the cell membrane to anoxia with release of potassium (252), and (ii) disintegration of the biochemical anatomy with freeing of the bound potassium from organic aggregates.

Following hypoxia, however induced, the sodium pump becomes ineffective. Interference with the cellular energy metabolism is proposed to be the prime factor responsible (169). Although endogenous substrates such as glucose can supply some energy anaerobically, the stored soon become depleted with accumulation of acids and decline in the intracellular pH (162). The alteration in ionic strength in the cytoplasm results in the entry of water and swelling of the cells and intracellular organelles. With breakdown of the energy supply to the cell wall, the membrane becomes permeable - sodium and potassium enter the cell, and magnesium is released (169). The increase of osmotic pressure resultant upon the entry of sodium leads to autolysis and an increase of intracellular molecules as synthesis ceases and waste particles accumulate (162). Swollen mitochondria are unable to perform oxidative phosphorylation after depletion of the respiratory cofactors NAD and ATP, and inorganic phosphate accumulates. The final stages of autolysis result from release of lysosomal enzymes, precipitation of protein, lysis of cell membranes and release of gylcogen and large intracellular particles.

A major contribution to the further understanding of the process of biochemical disintegration has come recently from the laboratory of Sir Hans Krebs (33). Original studies had shown that the ratio

$$\frac{\beta\text{OH butyrate} \times \alpha\text{ oxoglutarate} \times NH_4}{\text{acetoacetate} \times \text{glutamate}}$$

is constant in the normal rat liver. In ischaemia, with the accumulation of ammonia, acetoacetate transforms to β OHbutyrate and the pH decreases due to increase in lactate, with hydrolysis of ATP leading to the disappearance of bicarbonate and an increase in pCO2. Under conditions of hypoxia, when the capacity of the urea cycle is impaired, the liver disposes of ammonia as follows:

(i) $NH_4^+ + NADPH + H^+ \longrightarrow$ glutamate + NADP-H20

(ii) glutamate and pyruvate $\longrightarrow$ α oxoglutarate + alanine. This group of experiments highlights the physiological role of α oxoglutarate, which will be noted again in discussion of other studies.

(c) Reversibility

This most important factor is ill-defined although several attempts have been made. A marked suppression of aerobic metabolism in rat liver slices was noted after an interruption of

blood flow for 15 minutes (286), but recovery was rapid. Longer periods of interruption led to further suppression with a slower recovery. A decrease in pH and loss of potassium (169) were also noted after temporary interruption of blood flow, and the release of magnesium and β glucuronidase was noted to precede a rise in aspartate transaminase (169).

In another approach to the problem of reversibility, Dawkins et al (58) attempted to define the alterations occurring in the liver during autolysis, and more importantly, investigated the value of provision of substrates to protect oxidative phosphorylation. From these experiments emerged "a pattern of the complex relationships upon which depended the life and death of the liver cell". The levels of glutathione were shown to be reduced initially, preceding a fall in oxidative phosphorylation, and accompanied by a fall in ATP and NAD. Oxidative phosphorylation had been chosen as the index of viability since it had been shown to decline first during autolysis (58). It was found that oxidative phosphorylation could be maintained by the addition of α oxoglutarate, and was dependent upon the levels of oxygen and liver glycogen. The mechanism of action of oxoglutarate was not well defined since it appeared to act as more than a substrate and the activity was not replaced by L-glutamate, pyruvate and lactate.

Adequate levels of liver glycogen were achieved by continued feeding of the animals up to the period of operation. The administration of high concentrations of glucose and oxoglutarate were found incapable of substituting for glycogen in the liver cell.

It was concluded therefore, that there was a complex interplay of factors upon which the perfused liver depended. The most important factors were oxygen, adequate levels of ATP, glutathione, pyrimidine nucleotides and liver glycogen, and when these were depleted, the water and electrolyte balance of the cell and its mitochondria became disturbed.

(d) The duration of ischaemia and the value of histology

These factors have been investigated by a number of workers but their observations vary widely. Recent evaluation by electron microscopy has extended this range even further.

The period of maximum tolerated ischaemia has been variously estimated according to the species studied and to the type of preparation used (256). A period of 20 minutes normothermic ischaemia due to inflow occlusion to the dog liver was found compatible with subsequent survival (215) but after 30 minutes, an increase in circulating levels of lysosomal enzymes was found, and 60 minutes was reported maximal for reversal of potassium efflux (252). After a half hour's normothermic anoxia, the perfused pig liver was found incapable of clearance of ammonia or BSP, or the regeneration of ATP (275), yet other workers have noted that "the majority of liver functions survive three hours anoxia with almost undiminished efficiency" (273), and animals have been found to survive after four hours ischaemia in an autograft model (97).

The value of histology - The normal morphology of the liver has only recently been more fully described with the demonstration of zonal differences to histochemical reactions (159). It has been suggested that residual enzyme activity may remain despite complete disintegration of cellular structures, and that mitochondrial systems may have a more resistant enzymatic activity than microsomes (245).

Significant changes as a result of ischaemia were not noted by light microscopy until after 4-6 hours (97) and despite the fact that permanent vascular damage resulted from ischaemia of longer than 60 minutes, no acute changes were seen; a moderate cellular degeneration occurred but no peri-portal changes were observed. After 2-4 hours ischaemia, lesions were noted in the sinusoids and outflow tracts of the liver, but not in the portal triad.

However, by electron microscopy, centrilobular loss of glycogen was noted after 45 minutes' ischaemia and mitochondria were noted to be swollen and poorly stained after 60 minutes.

Longer ischaemia resulted in centrilobular fatty degeneration and hydropic changes in parenchymal cell cytoplasm, and ultrastructurally, profound changes were noted in the sinusoidal epithelium with increase in the size of Kupffer cells and their granules which might obliterate the sinusoidal lumina and the space of Disse. Dilatation and vacuolation of the endoplasmic reticulum was noted with decrease in the number of heavy aggregates and increase in the lighter, with resultant decrease in RNA and protein synthesis. These changes were shown to be reversible after 4 hours (216).

The temperature under which ischaemia or hypovolaemia is sustained is critical. In perfusion studies (58) it was shown that between 35 and 43°C, there existed an optimal temperature for oxidative phosphorylation in the rat liver. There also appeared to be a direct correlation between temperature and the rate of accumulation of calcium, which was also related to the decline in oxidative phosphorylation - for example, glutathione levels were better sustained at 35 than 38°C. Hypothermia to 30°C resulted in release of potassium without glucose, whilst with further reduction to 15° both were liberated (101). In studies using the slowly cooling liver to evaluate the effects of temperature, van Wyk et al (275) showed that even a slight decrease in temperature afforded a marked protection to function as assessed by perfusion. Reduced blood flow also accompanies cooling (149).

iii) **Methods chosen in this study**

Introduction

It is difficult to compare the physical and biochemical characteristics noted by other workers. This is compounded by the fact that, in many experiments, human blood has been

used without comparison with homologous perfusions. In addition, few studies exist in which the function of the isolated organ has been compared with that of the intact animal (74,268), and hence evaluation is valid only when comparing one perfused liver with another, and one series with another, but not when comparing physiological values relating to the intact animal. The few studies comparing liver function in vivo and in vitro demonstrate a major deficit in the function of the isolated liver.

A great deal has been written concerning the assessment of function of the perfused organ, especially in regard to the rat liver (9), but the studies reported here have been directed towards the clinical problem of hepatic assist. Hence, a range of functions has been used to give a broad spectrum for assessment. These have been used with varying frequency during perfusion and hence will be briefly described prior to the presentation of each group of experiments. After this presentation, reasons will be given for alterations in assessment. Based on the information outlined above, the experiments to be described have been assessed as follows:

(a) Physical characteristics

(i) Resistance - the ratio of pressure either in cm H20 for the portal system or mm Hg for the arterial system, divided by the flow rate in ml/g/min for each system provides a better index of the response of the liver to the flow offered, than do pressure or flow alone. The results are expressed in arbitrary peripheral resistance units for which the quoted maximal values are 0,02 in the portal system and 1,0 in the arterial system (192). Factors influencing resistance have been carefully evaluated in the dog liver and include histamine, endotoxin, acidosis, adrenaline, noradrenaline, temperature and hypoxia (69). However, the mode of action of most of these substances is thought to be via the "outflow block" which bedevils experiments with the dog liver where spasm of prominent sphincters in the hepatic veins results in total cessation of flow (69). These sphinters are poorly developed in the species under review and hence most of these factors do not interfere with perfusion.

(ii) Total flow - It has been suggested that the optimal flow rates for the liver are from 0,5 to 1,0 ml/g/min (61) but that these may be reduced if flow takes place through both the portal and arterial systems (61). The total flow rate to the liver has been variously calculated (268). In experiments using direct cannulation of the vessels with the liver still in situ, and with minimal anoxic time, flow rates of 0,6 ml/g/min were obtained without excessive pressures (168). A factor of great importance is the inherent regulation of flow for example after digestion. Certainly it has been demonstrated in studies of the isolated frog or rat liver, that individual areas of the liver are not all perfused simultaneously, but that regulation by sphincters results in intermittent filling and emptying. It has been suggested that intermittent changes in size of

the reticulo-endothelial cells are the basis of this regulation of blood flow (185). Further regulation of sinusoidal distension may be achieved by outflow vascular resistance which is thought to promote maximal blood/hepatocyte contact (274). In the experimental design, this may be achieved by siting of the vena caval catheter so that intermittent filling and emptying of the liver results. However, the pressure in the vena cava must be monitored carefully, since in some instances, high resistance to caval drainage resulted in the development of outflow block. In the same experiments it was felt that a positive outflow pressure improved bile flow rate (56). Certainly it is generally accepted that increased outflow tract pressure increases the rate of ascites formation (190).

It has been questioned whether a pulse is needed in the arterial system - indeed, whether perfusion of the arterial system is necessary at all. In the absence of pulsation, an increase in pressure and reduction of flow into the hepatic artery was noted (187), whilst in the total absence of arterial supply, it has been observed that biliary secretion rate drops (65) and that citrate cycle metabolism is less effective (271).

Optimal division of the flow rates has been quoted to be 0,32 ml/g/min through the arterial system, and 0,65 ml/g/min through the portal system, this latter being 85-90% saturated with oxygen (129).

(iii) Pressures - Arbitrarily, but based on observations made in random animals during anaesthesia and prior to dissection of the liver, the maximal pressures accepted in this study were 15 cm H20 in the portal system and 100 mm Hg in the arterial system.

(iv) Bile flow rate - In the experiments to be described, initial studies were performed to evaluate the rate of flow and the trend in secretion rate during perfusion without the addition of any form of choleretic. Thereafter, since biliary secretion rate in the pig has been shown to be dependent upon bile salt load, the circulating secretin level and neurogenic stimuli (104) and since the action of the former substances lasts only 1½ to 2 hours unless replenished, arbitrary amounts of bile salt and secretin were added to perfusions as a continuous drip infusion. The bile salt selected was deoxycholate which is the principal acid secreted in the pig bile (105); this was obtained as the intravenous preparation of the sodium salt - Decholin (BDH). Bile flow may also be stimulated by cholic acid (289).

Investigation of the effect of hypertonic solutions upon bile secretion (44) has resulted in the proposal of two mechanisms of secretion - osmotic activity leading to water and electrolyte excretion, and active secretion of bilirubin and bile acids. It is also suggested that the action of secretin has been to produce a fluid rich in bicarbonate and poor in chloride, or a fluid with fixed electrolye composition unrelated to ion exchange or reabsorption within the biliary tree (102). The bile salts are supposed to act as osmotic diuretics with reduced chloride and

bicarbonate levels (104). As a result of this confusion surrounding biliary secretion, a great deal of work is necessary to elucidate precise mechanisms (76). From the numerous regulatory mechanisms however, it is obvious that the measurement of bile flow alone without evaluation of the quality and composition of the secreted fluid, is not an indication of function but rather of substrate availability. Similarly the wide ranges of flow rate reported from different centres may relate to the added use of choleretics.

Haemobilia as a result of arterial pressure greater than 150 mm Hg or a total flow greater than 1,0 ml/g/min, clearly indicates gross damage (61).

(b) Biochemical characteristics

Many tests have been suggested for the function of the perfused liver (3,2). In this study, functions were chosen which gave evidence of - cell membrane integrity, viz. electrolytes and oncotic pressure; - the provision of energy from glucose and lactate metabolism; - the acid base status and oxygen consumption; - damage to liver parenchyma.

— The evaluation of cell membrane integrity has been made in relation to the known factors in ischaemia and necrosis as described previously. Elevation of plasma potassium and increase in osmolality were taken to suggest greater membrane permeability with leakage. More recently, direct measurement of membrane potential has been suggested by Lambotte (147,148) in which maintenance of the mean potential difference resultant upon passage of a probe through at least 50 cell membranes has shown good correlation with results of reimplantation of such organs.

— Interpretation of the metabolism of glucose has been complicated by lack of knowledge of the in vivo and perfusion relationships. The results of study of glucose metabolism are conflicting - many workers propose that a rise in perfusate level during experiments indicates synthesis of glucose (136), perhaps from gylcogen or lactate. Other workers report uptake of glucose by the liver (198) but the interpretation of these conflicting results is uncertain. Contributing facts may be (a) different conditions of starvation preceding the procedure; (b) a wide range of perfusate glucose levels; and (c) the reduction of levels of insulin and glucagon in the perfusate due to the short half-lives of these hormones, and to their destruction by the trauma of oxygenating systems (163). Again two divergent reports exist concerning insulin metabolism - one suggesting no significant effect of insulin on glucose metabolism by the perfused liver (183) and another in fact demonstrating an effect if transhepatic measurements were made (126). Regulation of glucose metabolism has been shown to be dependent upon the breakdown of glycogen into glucose, the entry of glucose into the cell under the influence of insulin, and the availability or high energy phosphate to catalyse anaerobic phosphorylation of glucose to intermediary compounds and pyruvate. Thereafter aerobic oxidation occurs of

pyruvate via the Krebs cycle with transfer of electrons by respiratory chain enzymes within mitochondria for oxygen consumption. In addition, the liver can synthesize glycogen or glucose by reversal of lactate/pyruvate metabolism (60).

— Lactate/pyruvate metabolism has been measured according to the recommendations of Huckabee (123,124) since the ratio gives a better index of cellular oxidation and aerobic metabolism as the reaction is coupled to the redox status of the NAD/$NADH_2$ system by the action of lactate dehydrogenase. Assessment of lactate metabolism in the intact dog showed a negligible rate in the resting state (95) but variations with different oxygen saturations have been noted (129) - 0,11 ml/g/min with 100% saturated blood, and 0,22 ml/g/min with 50% saturated blood. The clearance of lactate has been proposed as a sensitive index of the adequacy of perfusion and since α oxoglutarate has been noted to rise with the administration of lactate, a maximal rate of metabolism has been postulated (270).

— The isolated liver should provide an admirable basis for the study of the role of the organ in acid base metabolism, but since no comparative in vivo studies are available, the significance of alterations in acid base status is unknown. The pH of the perfusate is maintained arbitrarily at 7,4 by the addition of sodium bicarbonate since a trend towards acidosis has been associated with increase in circulatory resistance (5). It may be that a slow trend towards acidosis should represent accumulation of organic acids resulting from metabolism, but the buffering capacity of the liver is not known.

— Oxygen consumption has been proposed to be a single, reliable guide to function (3), but when the blood flow is slow or static, the rates of consumption may be falsely elevated due to the longer availability of blood for extraction of the oxygen.

— Damage to liver parenchyma has been assessed by the measurement of aspartate transaminase which has not been used much by other workers, presumably because of the associated damage to red blood cells in oxygenation and pumping, with release of the enzyme. However, in these studies, the plasma haemoglobin level has always been related to changes in enzyme level. Damage to the liver has been reported to occur during hepatectomy (12,193), but this accumulation should be rinsed out during flushing. In addition, an intra-operative period of recovery from "unconventional ischaemia" has been proposed by Abouna (3), and it has been suggested that adequate perfusion may reverse damage which has occurred during hepatectomy (103). However, during perfusion, no extra-hepatic regulation can occur of the mechanical pumping of arterial flow into the liver and overperfusion and damage are likely to occur.

(c) Biliary secretion

Apart from the observation of flow rate, plasma alkaline phosphatase and cholesterol, biliary electrolytes and osmolality were also measured. However, the relationship of biliary electrolytes and osmolality to those of the plasma is not known. Whilst the integrity of cell membranes could again be invoked to explain differences in electrolyte levels, the role of active transport and secretion has yet to be elucidated for the biliary system as has been expounded for the renal system.

Plasma alkaline phosphatase and cholesterol were selected as having clinical significance and should be of value at least in confirming evidence of biliary stasis. However, in perfusions of this short duration, alterations were small and insignificant.

(iv) **Excretory characteristics and clearance**

These have not been evaluated in this series of perfusions. In other work, indocyanine green, Rose Bengal (289), bilirubin and BSP (156), have been used to determine clearance. BSP has not been used by some workers as a single bolus dose (156), since it was found to be rapidly removed before adequate mixing could take place. Although isolated liver physiology has been evaluated by the use of BSP (42), neither BSP nor ammonia clearance was found as sensitive to changes in temperature as lactate clearance (257). More recently, however, it has been suggested that the BSP transporting ability is a more sensitive index of liver cell damage than the pure measurement of BSP retention (3) and the isolated organ is ideal for this measurement since there is ready access to the bile.

Further evaluation of the mechanism of clearance has suggested that a functional division may exist (79) - parenchymal clearance of galactose and lactate, hepatobiliary clearance of BSP, bilirubin and Rose Bengal, and mesenchymal clearance through the Kupffer cells of various particles. It is now thought that there are two components to clearance - removal of the substance by the liver, and storage within the cells with subsequent release (284). Galactose elimination has been suggested to be independent of bile and blood flow and to be representative of functional cell mass (128).

(v) **Synthetic ability**

Sir Hans Krebs has recently stated that the only true means of evaluation of the function of the liver is in the study of its synthetic ability (145) and indeed sophisticated studies in the rat liver have been performed in that laboratory (111), and by Exton and Park (77) and many others, but these belong to the realm of the physiologist. The studies to be described benefit from the results of these sophisticated investigations, but in the interests of practicability, such work does not have direct application in this clinically directed programme.

(vi) **Reimplantation**

Reimplantation, however, constitutes the only true method of assessment of total liver function, but due to the complexity of the procedure, only a few such studies have been performed. Kestens (136) has reported survival after perfusion at normal temperatures for periods of 6-8 hours. This technique holds the key to the solution of the coagulation problem, since in the perfusion system due to the presence of heparin, few adequate studies have been made of coagulation factors.

CHAPTER THREE

A. PREPARATION OF THE LIVER

i) Animals - pigs, lambs and calves, baboons

ii) Preparation - (a) pre-operative
(b) anaesthetic techniques
(c) intra-operative fluid

iii) Operative technique

iv) Blood and blood grouping

v) Anti-coagulants

B. THE PERFUSION CIRCUIT

i) General

ii) Oxygenator

iii) Gas

iv) Pumps

v) Tubing

vi) Other

vii) Sterility

viii) Liver weight

ix) Technique

x) Flushing of the liver - (a) constitution
(b) pressure
(c) volume
(d) temperature

xi) Priming solutions and additives

A. PREPARATION OF THE LIVER

Probably the most important and least studied contribution to the success of liver perfusion lies in the selection and surgical preparation of the donor animals. Hence, the following section is dealt with in detail.

i) Animals

In the original design of the study, it was decided to commence the investigation using pigs. The porcine liver had been used in the majority of clinical hepatic assist procedures reported, and hence seemed suitable. In addition, recent knowledge and experience in the handling of the animal had been obtained by one member of the Liver Research Group.

Pigs have generally been selected for use in such programmes because they are cheap and easy to maintain (64); the liver is free from bacteria, and, perhaps the most important factor, they do not manifest the "outflow block" which has complicated research using the dog liver. The response of hepatic vein sphincters which are poorly developed in the pig, is practically never seen, although it is suggested that in isolation, some venous obstruction may result in the presence of acidosis (274). Perhaps the only disadvantage to the use of the pig liver is the lack of biochemical information available especially for the age of animals used. Hence a study was made during initial experiments in a transplantation programme, in which normal biochemical, haematological and organ weight values were collected (116,119).

With subsequent extension of the perfusion project, it was decided to use baboons, calves and lambs. Little work has been published on perfusion of the isolated baboon liver since the animals are not readily available in other countries; sheep livers have been used in a variety of physiological studies (156) and the calf liver has been used both for physiological and clinical application (26).

Pigs were obtained from two farms in the Western Cape from the general market stock. They were transported aged about four weeks just after weaning, and were not castrated. The breeds used were Landrace, and cross Landrace/Large White. The optimal weight for handling was 15-20 Kg, and such animals provided livers weighing between 540 and 720 grams, which were suitable for the design of the circuit and the priming volumes used.

Lambs and calves were obtained from farms at random and were selected to be as small as possible to provide comparable organ weights.

Baboons (of the Cape Chacma type, Papio ursinus ursinus) were obtained by courtesy of the University of Stellenbosch Primate Facility at the Karl Bremer Hospital. These were used according to available size - ranging from 7 to 15 Kg.

All animals were maintained on standard rations for the duration of their stay - pigs

received a commercial ration (Creep meal) which contained recommended ratios of carbohydrate, fat and protein with supplementary vitamins and minerals. Calves and lambs, being very young, were procured the day before use and hence needed little feeding. Baboons received a standard vegetable diet.

ii) **Preparation**

(a) Pre-operative: Except in experiments where otherwise stated, all animals were starved for 24 hours prior to surgery. This was of particular importance in regard to the pig where the bowel occupies a large area in the abdominal cavity and renders dissection and retraction almost impossible if filled with solid pultaceous faeces. The pig also appears to have a slow gastric emptying time and hence required starvation for the minimum of 24 hours. Animals were offered 5% dextrose water but in general did not take this. Further investigation of the possibility of feeding a liquid diet is necessary since it has been clearly shown that various aspects of liver function are protected in livers in which there is an adequate store of glycogen (58).

In animals which were to be used for clinical hepatic assist, antibiotics were given - chloromycetin, penicillin and streptomycin which covered the flora cultured at random in the laboratory. If insufficient time was available before the dissection was undertaken, penicillin and rolitetracycline (Reverin) were given intravenously during removal of the liver.

(b) Anaesthetic techniques: These varied with the different animals used.

Pigs: Initially halothane anaesthesia was used with great success, but after interruption of the programme by the complication of malignant hyperpyrexia which occurred in 25% of the Landrace pigs used (107), this drug was abandoned in favour of the injection of sodium Pentothal into an ear vein. No premedication or sedation was used and during injection the pig was manually restrained. Usually 10-15 ml of a 0,5% solution injected over 2-3 minutes allowed ample time for weighing and positioning of the animal on its back for insertion of an endotracheal tube. This was achieved under direct vision by standard techniques (260) using a Magill laryngoscope with xylocaine spray to the vocal cords. Anoxia was encountered on only two occasions and tracheostomy was never employed. A wide bore Ryle's tube was passed also under direct vision, to provide drainage of any residual fluid in the stomach.

The endotracheal tube was connected immediately to a mechanical (Cyclator) ventilator with a closed Magill-type circuit. Pressure and rate of ventilation were adjusted to those observed in the animal and were controlled by blood gas analysis. Initially, attempts were made to continue the abdominal operation under nitrous oxide/oxygen anaesthesia alone, but the muscular relaxation was inadequate, and δ tubocurarine 1,5 mg was injected as soon as an

intravenous line was established. This relaxant was sufficient for the duration of the operation and was not reversed; however the drug was not used in preparations for clinical procedures.

Baboons: These animals were immobilised in a "squeeze cage" and sedated with an intramuscular injection of Ketamine 5mg/kg. The duration of action of this drug was 20-30 minutes, but within 10 to 15 minutes, the animal was relaxed and could not bite. An endotracheal tube was passed under direct vision and initial anaesthesia was maintained with nitrous oxide, oxygen and halothane. As soon as the intravenous route was established a similar dose of curare was injected. In general a stomach tube was not required.

Calves and lambs: These animals were induced with halothane administered through a nose cone, and anaesthesia was subsequently maintained with nitrous oxide, oxygen and halothane. Relaxation with curare was also employed.

(c) Intra-operative fluid: Replacement fluid took the form of 10% Invert Sugar in Ringer lactate - 300 to 500 ml being given during the procedure according to the size of the animal and the blood loss. In addition, in later experiments, as soon as the intravenous route was established, 25g of dextrose was given to restore hepatic glycogen stores after depletion by starvation, and to prevent total depletion by the effects of adrenaline stimulated during surgery.

Total body heparinisation was achieved by the administration of 2mg/kg just prior to final removal of the liver.

iii) **Operative technique**

In general the operative procedure was similar in all animals, but certain differences will be highlighted.

The internal jugular vein was cannulated with a Portex umbilical feeding tube (Fg 9) as an initial procedure to provide an intravenous route for drug administration. This was particularly important in the pig which has small peripheral vessels. For convenience an arterial pressure line was established through the same incision, in the common carotid artery. This consisted of a short length of intravenous infusion tubing attached directly to a "Tycos" pressure gauge; this line could be filled and flushed with heparinised saline and resulted in a very efficient monitoring apparatus.

A midline abdominal incision was chosen as the most practicable although the possibility of a subcostal transverse incision was also explored. This latter resulted in considerable blood loss and did not materially improve the exposure. As these animals all have long narrow chest cavities, the livers tend to be high up under the costal margin, and when necessary, the chest was opened.

Dissection commenced around the left diaphragmatic border of the liver with incision of the triangular ligament and incision into the lesser sac. The hepatic artery of the pig lies to the left of the portal vein and hence can easily be felt and dissected (see Figure 3). The first branch to the stomach was ligated and the dissection continued down to the pancreatico-duodenal branch of the coeliac which was also ligated. During this and subsequent dissection, it was found important to incise and leave open any lymphatics. The pig periportal and peri-hepatic arterial tissue is particularly rich in lymphatics and it was found that ligation or cautery of these resulted in oedema of the porta hepatis which often became gross and was thought to contribute to oedema around the intrahepatic portal radicles with resultant rise in portal pressure. At this stage of the procedure, the peri-portal tissues were approached and freed from surrounding structures. The bile duct was identified and dissected from the junction of the cystic and hepatic ducts down to the entry into the duodenum, which in the pig is only a few millimetres distal to the pylorus (247). The duct was ligated just above the duodenum and a catheter 4mm in diameter was inserted for drainage of bile as an intra-operative collection. This sample was drained for an hour and served as the pre-perfusion control. The cystic duct was immediately dissected and ligated to prevent drainage from the gall bladder. The cystic artery was preserved. In further dissection of the portal vein, a large constant lymph node was found on the right side and was removed with a number of smaller inconstant nodes which seemed more prevalent in younger pigs. The pancreatico-duodenal tributaries of the portal vein were ligated and the portal vein was dissected free down to the confluence of the superior mesenteric and splenic veins. At this point it was possible to palpate the hepatic artery dorsal to and on the left of the portal vein and access to it could be more easily obtained by retraction of the vein to the left. Hence the artery could be freed from the branches of the coeliac axis. Depending on the calibre of the vessel, dissection was limited to the provision of an adequate length for cannulation. In the baboon, it was necessary to include a segment of aorta in dissection, since the calibre of the hepatic artery was insufficient for effective cannulation.

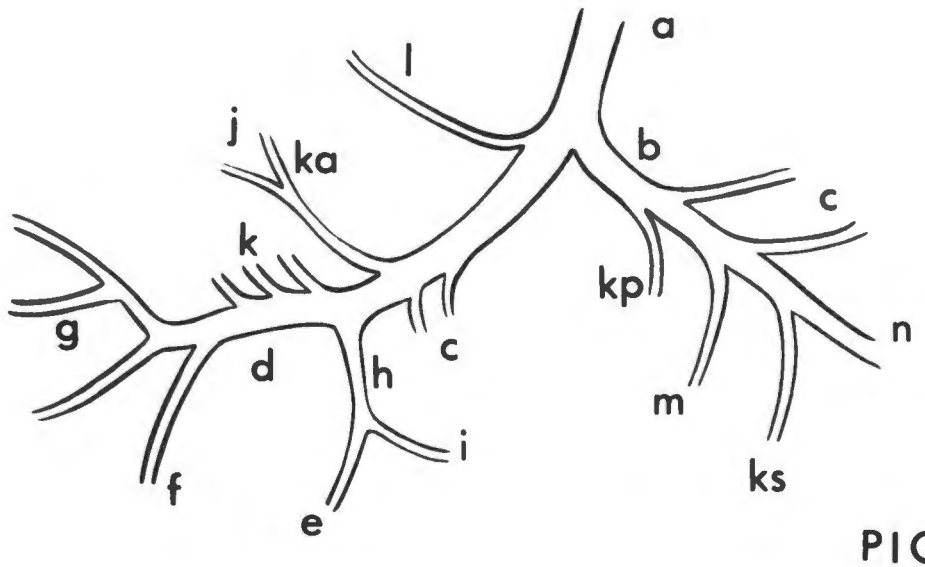
Attention was next directed to the dorsal surface of the liver. In the pig, the infra-hepatic cava is extremely short before it is covered by a tongue of liver, and hence careful dissection has to be made of the liver from the diaphragm. However, the liver is much less adherent to the diaphragm than that of the baboon. This adherence was more marked in the younger animals and resulted in difficulty with the baboon liver which seemed more friable.

The phrenic veins were next ligated and the liver was totally free of all attachments except a full vascular supply. At this stage the animal was heparinised and a short recovery period of 30-45 minutes was allowed while the circuit was primed and blood and diluting solution were mixed and warmed, and while blood gas analyses were performed. Final removal of the liver commenced with ligation of the portal vein above its origin, and insertion of a length of Silastic

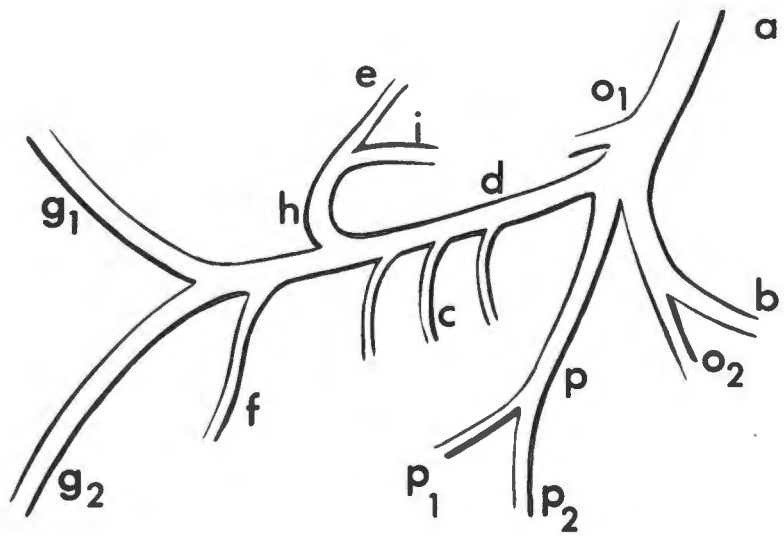
Figure 3 : The hepatic arterial anatomy of the pig, calf and lamb. The position of the liver in the calf and lamb is shown in the lower diagrams.

Key:

a) coeliac
b) splenic
c) pancreatic
d) hepatic
e) R. gastroepiploic
f) cystic
g) hepatic branches - (g 1) ventral (g 2) dorsal
h) gastroduodenal
i) pancreatico-duodenal
j) oesophageal
k) gastrics (ka) anterior (kp) posterior (ks) short
l) to left crus of diaphragm
m) arteria diverticuli
n) L. gastroepiploic
o) ruminal (o 1) left (o 2) right
p) omaso-abomasal (p 1) ventral (p 2) dorsal



PIG



CALF & SHEEP

tubing 6mm diameter. Through this catheter infusion of the cooling and flushing solution was begun. As infusion continued, the infra-hepatic vena cava was clamped and the diaphragm around the supra-hepatic vena cava was incised. The supra-hepatic cava was traced up towards the right atrium and was clamped as high as possible. The cava was incised to release the infusion fluid which had accumulated in the liver. If large enough, the hepatic artery was cannulated directly with a Portex umbilical feeding tube (Fg 9), and infusion commenced immediately. If too small, the artery was closed with a bulldog clamp and the segment of aorta was removed. The lower cava was cut and the liver was removed to a bowl for collection of the flushing solution and cannulation of the remaining vessels. The vena cava above and below the liver was cannulated with a Y-shaped tube since it had been found beneficial to drain both ends of the cava; if the lower end were tied or included a thermometer probe, the small caudate lobe became markedly distended. These cannulae were inserted allowing a short segment of vessel free above the exit to allow the intermittent filling and emptying which was thought to simulate cardiac action and the intermittent negative pressure on the sinusoids. If the hepatic artery had not been cannulated in the animal, the aortic segment was ligated and all air excluded by infusion of fluid through the Portex umbilical feeding tube which was then tied into the vessel and the bulldog clamp released to allow flushing of the hepatic artery which was continued under slight pressure from the intravenous infusion set.

The liver was then transported to the perfusion laboratory where it was inserted into the circuit after initial preperfusion samples had been taken. In all cannulating procedures and with connection into the circuit, great care was taken to eliminate air since this resulted in extremely high resistance particularly in the hepatic arterial system. The final temperature of the organ was recorded just prior to the institution of perfusion. When the perfusate temperature was 4°C, the liver arrived in the laboratory at a mean temperature of approximately 12°C, whereas when the perfusate temperature was 37°C, the final recording was approximately 26°C.

Dissection of the calf or lamb liver differed slightly in that the organ lay entirely to the right of the abdominal cavity being displaced from the left by the stomach (see Figure 3). The portal vein was short and covered by the pancreas, with many small pancreatic tributaries which hindered dissection. The portal vein was directed towards the left at the porta hepatis as the left gastric vein formed a large tributary. The intra-hepatic cava was more inaccessible than in the pig as the whole plane of the liver was tilted towards the right hence covering the area of dissection. In addition, the vena cava was more retro-peritoneal than in the pig and required more dissection.

iv) **Blood and blood grouping**

Pigs - have a number of blood groups of the minor type (179). Following much experience with a transplantation programme in the pig, it became evident that no cross-matching of blood between these animals was necessary. No allergic response was ever seen in the numerous instances of infusion of abattoir blood to liver transplant recipient animals. However, it has been shown that pig erythrocytes cross react with human sera and that the pig can thus be "grouped" into A, B and Ohuman types. For the purposes of the experiments to be described, only the A grouping was made since the preparation of the anti-B test serum requires a project of its own. Animals were thus designated A or O according to the reaction of erythrocytes with a specific human anti-A serum, prepared according to the method of Lockyer (157) by absorption of heterophile antibodies with pig group O erythrocytes.

Porcine blood for homologous perfusions was obtained fresh on the morning of the experiment from the abattoir. Animals among the general market stock were initially stunned and then hoisted by a chain around the hind limb on to a conveyor belt. Whilst the animal was thus suspended, the throat was slashed and the blood was collected directly into a 5 litre plastic container through a plastic funnel. Although this was relatively clean, no attempt could be made to collect blood under sterile conditions.

Baboons - secrete in the saliva and other secretions, substances analogous to the human blood group substances, and hence these animals were grouped as humans into groups A, B and AB. Blood group O does not appear to exist in baboons (176,285).

Calves and lambs - Since little information could be found concerning the blood groups of these animals, they were used ungrouped.

Human blood for heterologous perfusion was obtained fresh from the Blood Transfusion Service on the morning of the experiment. Initially collection was made into glass bottles, and the subsequent change to plastic packs seemed not to alter the platelet counts nor their response in perfusion.

v) **Anticoagulants**

Since citrate metabolism has been shown to be impaired in the presence of poor liver function (271), it was originally decided in the clinical management of patients with massive liver cell necrosis to select heparin as anticoagulant. Hence all experiments were performed with heparinised blood. While the standardisation of the dose added to human blood was easy (22,5mg/500ml), it was more complicated for the blood to be collected from the abattoir. However, it was felt important to ensure adequate heparinisation and hence 250mg was injected into the container which usually held 2-2,5 litres of blood. This final concentration of 0,4mg/ml was ten times that in the human blood.

In regard to the analysis of perfusion samples, the use of heparin was a complication in that fibrinogen cannot be measured in the presence of heparin, and high concentrations of the drug can interfere with the measurement of complement (90). However, the concentration used in any one experiment was constant as far as is known, and comparison was made of data within each experimental group.

B. THE PERFUSION CIRCUIT

i) General

The circuit was designed with the principal objective that it should be compact and portable for clinical application. In addition, with the original plan that such procedures might be applied in the ward beside the patient's bed, all parts of the apparatus were adjustable in height (Figure 4).

A single trolley was designed on which were carried the pumps, and the perfusion chamber, and which also provided electrical connections. As will be seen from the diagram, the pumps were sited to one side of the trolley with the oxygenator supported on adjustable rods which could be inserted into rings welded onto the base of the trolley. The chamber, 15" x 10" oblong and 10" deep had a sloping base down to an exit tube. The chamber was closed with a lid of glass and steel so that closed observation of the liver could be made. Holes drilled in the sides of the chamber allowed exit of catheters, and the lower exit tube drained ascites or any blood leakage. Bile was collected in a graduated tube within the chamber in order that the length of the draining catheter should not provide too great a resistance to bile flow. The liver was supported within the chamber on a diaphragm of soft fibre-glass gauze which was attached within the chamber by elastic hooks from each corner. Mobility was achieved in later experiments by attachment to a small motor with an eccentric wheel and adjustable speed (Figure 5). The excursion provided to the diaphragm was 5cm/cycle and the rate was set at 16-20 cycles per minute. Although no definite studies were performed, it seemed that this movement resulted in better perfusion of the posterior surface of the liver.

ii) Oxygenator (Figure 6)

In the original investigation of the available types of oxygenator, the bubble type was selected. The disc type, whilst perhaps superior in regard to its trauma to blood, was tedious to clean and expensive in requiring new discs to be obtained if damaged during use. The membrane oxygenator, whilst probably superior was both unavailable in the country and prohibitively expensive to import. The disposable bubble type (Rygg Kyvsgaard) was freely available and had been used for clinical and experimental work so that much experience was available. The smallest "Infant" type was used. A priming volume of 2½ litres was needed to continue a four-

hour perfusion during which a gas flow of 500-1 000ml/min was needed. Subsequent studies reported in more detail in a later chapter resulted in the use of a smaller oxygenator - the Travenol Miniprime bag which required a lesser priming volume of only 1,5 litres and a gas flow of 300-400 ml/min.

iii) **Gas**

In previous descriptions of perfusion circuitry, a 95% oxygen 5% CO_2 mixture was used. However, it was found that pure oxygen and carbon dioxide were more readily available locally than mixtures which had to be transported 1 300Km with inevitable delays. Hence a rotameter block was obtained by courtesy of the Anaesthetic Department, Groote Schuur Hospital, and individual gas rates could be used. A final oxygen rate of 300-350 ml/min and carbon dioxide rate of 30-50 ml/min were used, giving a final concentration of 91% O_2 and 9% CO_2. Final adjustments were made according to the blood gas analyses when the flow rates had been determined.

iv) **Pumps**

Individual Sarns roller pumps were chosen for their ability to provide relatively non-traumatic pulsatile flow. Although several workers have claimed successful perfusion with portal supply only, or with an arterial infusion by gravity from the main portal line (61), in this experimental model, as physiological a preparation as possible was sought, and hence the hepatic artery was perfused individually and under pulsatile conditions. Each pump was set at optimal calibration of rollers (recommended occlusion such that a column of fluid 36" high should fall less than 1" in 1 minute), and the individual stroke volumes were calculated according to the tubing used. These were measured at a number of different speeds and the results graphed. Subsequent flow volumes were calculated direct from the pump speed.

v) **Tubing**

In view of the diverse application of the perfusion model, it was thought preferable to use silicone rubber rubing (Silastic) throughout the circuit. All except the segment through the hepatic arterial pump was 0,625 cm internal diameter, and the arterial section was 0,25 cm internal diameter; this bore was selected to allow a smaller volume to be delivered by a relatively fast pump speed. For experimental perfusions, tubing was rinsed in cold water and then soaked overnight in warm Lux flakes. It was then left to dry. A Dow Corning medical grade silicone spray was applied to all non-tubing portions of the circuit after every 5 or 6 perfusions. In clinical hepatic assist procedures, as much disposable apparatus as possible was used and discarded, including tubing and connectors.

vi) **Other**

Connectors were limited in number in the circuit and were made of polypropylene (Portex).

Figure 4 : The lay-out of the circuit used for the perfusion of the liver in isolation.

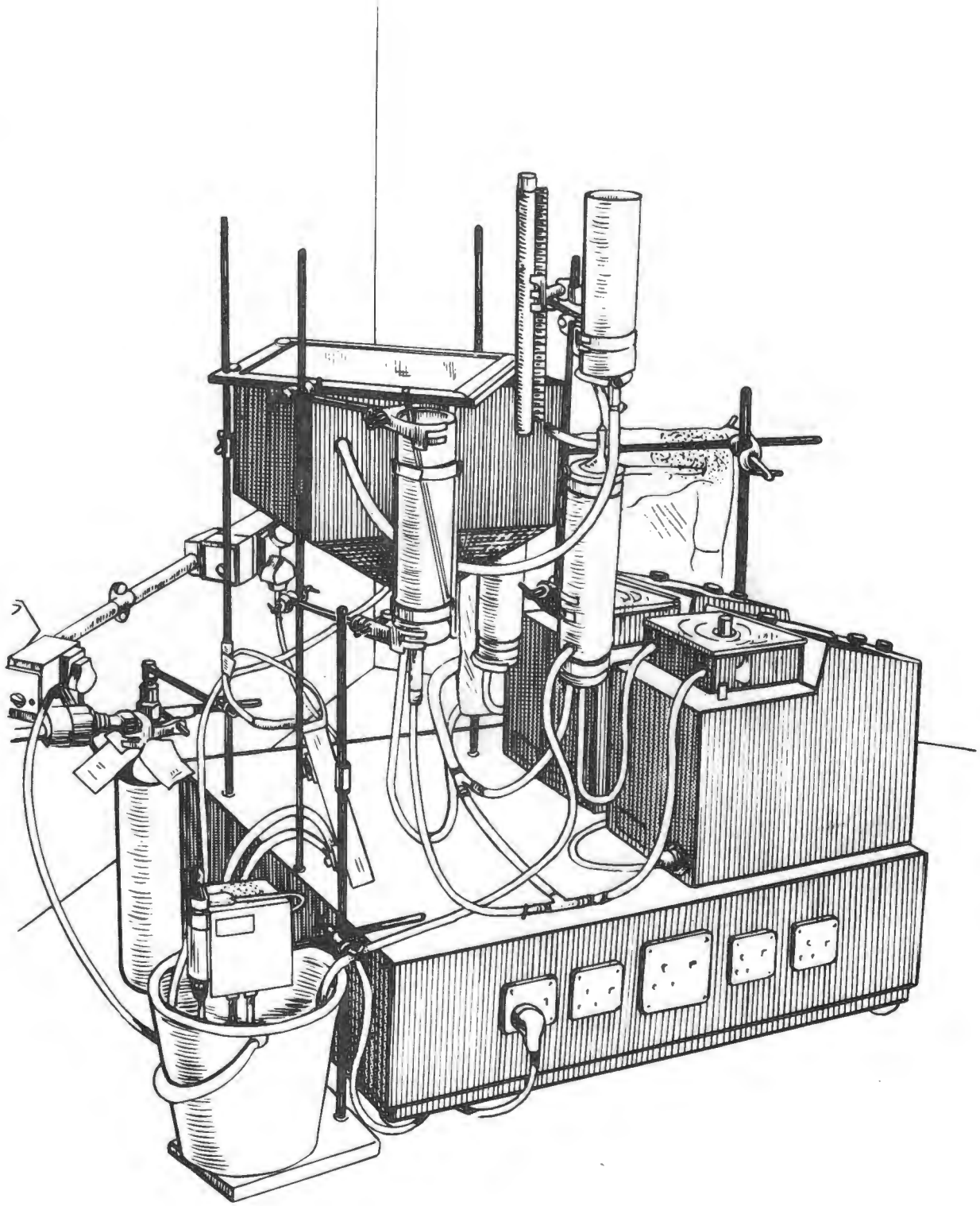


Figure 5 : A cross-section of the perfusion chamber and diaphragm motor.

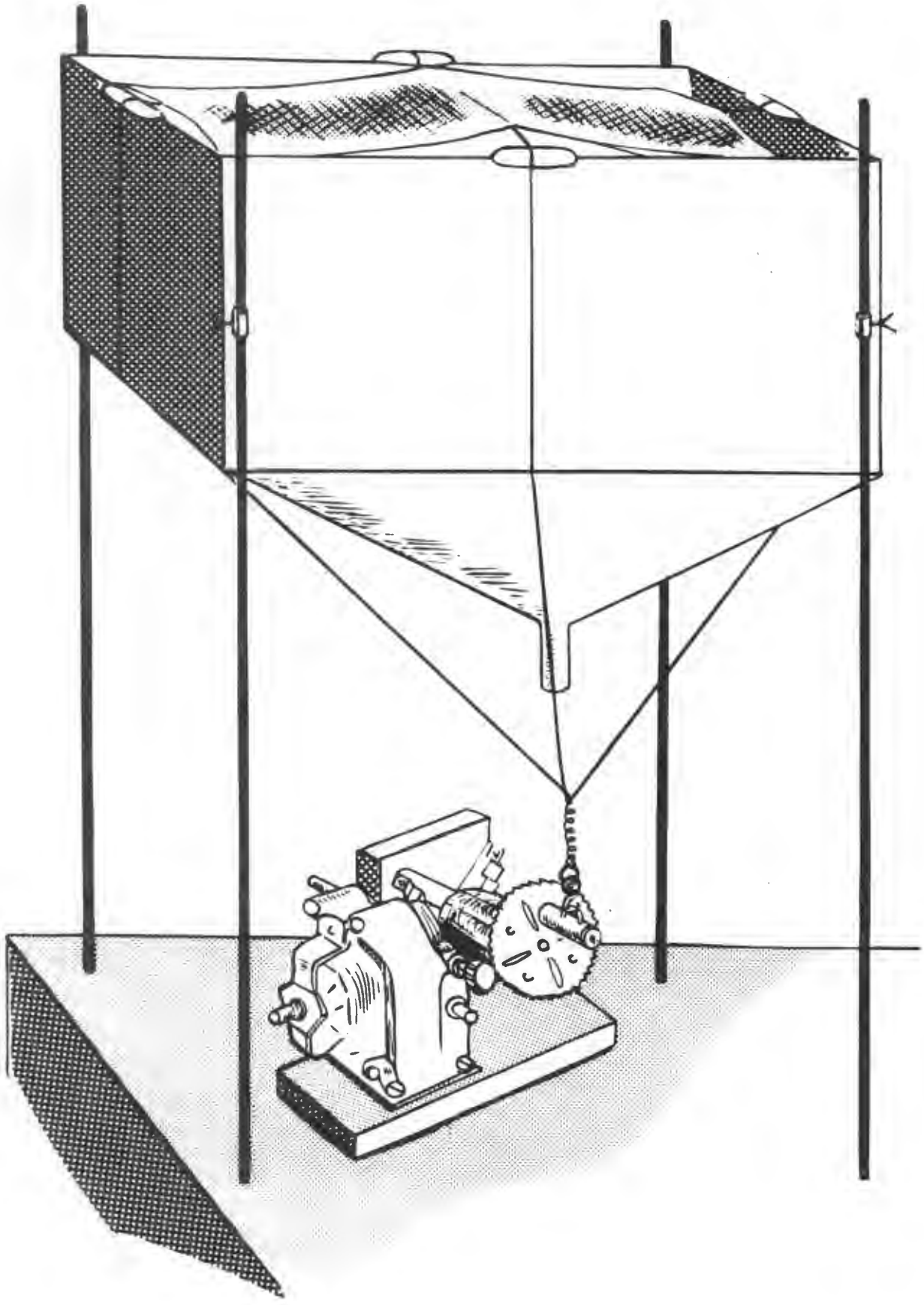
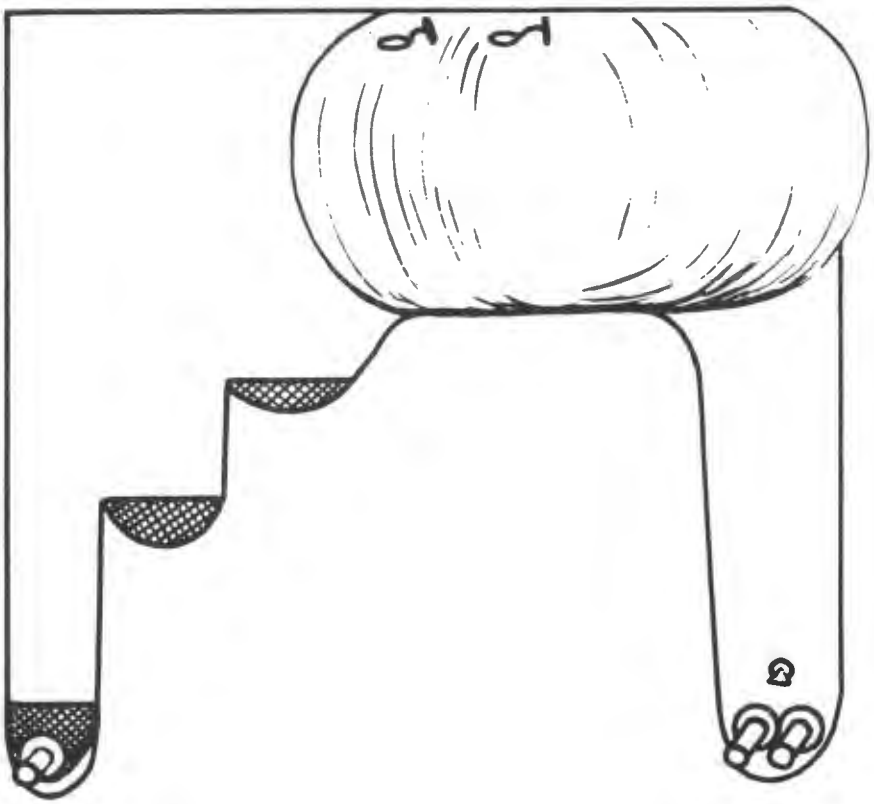
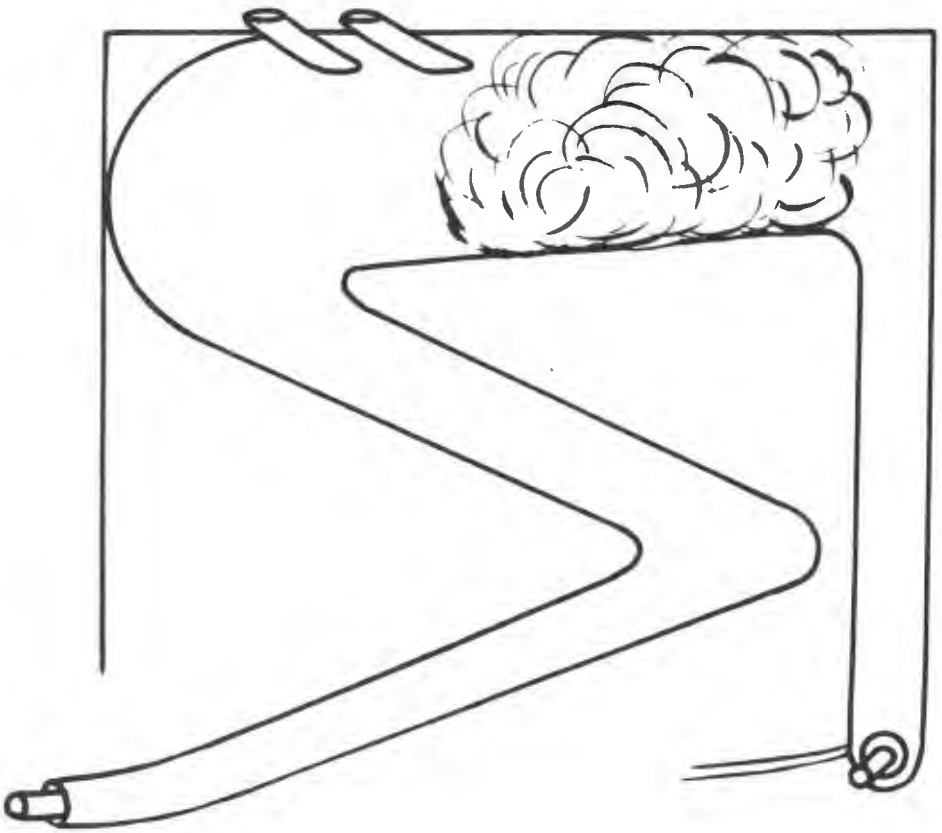


Figure 6 : Diagrams of the Rygg-Kyvsgaard and Travenol oxygenators.





A specific cannula for the double vena caval drainage was constructed with a T-piece and a Y (Figure 7).

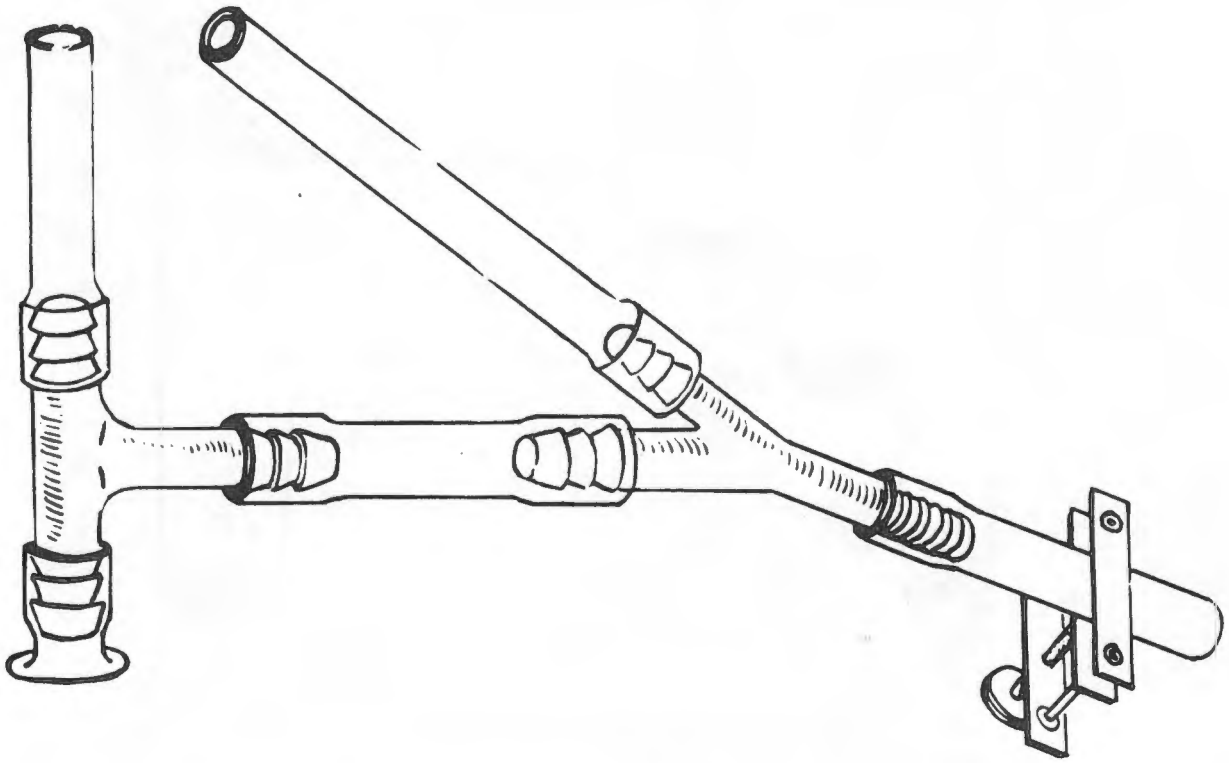


Figure 7 : The Y-piece inserted into the supra- and infra-hepatic vena cavae.

Heat Exchangers were constructed of highly polished stainless steel on the design of Brown Harrison (88) and heating water was delivered to the outside jacket by a thermostatically controlled laboratory water circulating unit at a maximum of 5 litres/min.

The final circuit is shown in comparison with that used for clinical perfusions (Figure 8). It was decided to use a similar circuit for clinical hepatic assist purposes for several reasons. (i) The technique when applied clinically is time-consuming and requires complete familiarity with the mechanics and circuitry and hence it seemed preferable to use the routine laboratory circuit. (ii) In the majority of clinical perfusions described by other workers, there has not been an oxygenator in the circuit, as the patient was connected directly to the liver and his own system was relied upon to provide oxygenation. However, it was felt that this technique was too hazardous in a patient with a failing circulation, or in a child with a small blood volume, and it was decided to incorporate an oxygenator into the circuit to ensure that an adequate volume of blood was available to supply the liver at all times. A similar inclusion is

being described more frequently in perfusion techniques. The circuit is shown provided the security of being able to isolate the liver and the patient with only two clamps without jeopardising either. However, as will be discussed later, the inclusion of the oxygenator could be questioned in the light of subsequent results.

In most previously described perfusions, there has been little measurement of the flow rate obtained from the patient, which rendered particularly difficult the assessment of the volume to be returned from the liver outflow. It was thus decided to allow the patient's blood to drain directly into a reservoir sited above the draining into the oxygenator, in which the flow rate could be measured. A Y-connection from the venous return line via a bubble trap and pump provided return to the patient. In this manner, the haemodynamic state of the patient could be more easily balanced.

vii) **Sterility**

Experimental liver perfusions were not performed under sterile conditions but for hepatic assist procedures, the system was boiled, autoclaved or gas sterilised, and the animals were given antibiotics.

viii) **Liver Weight**

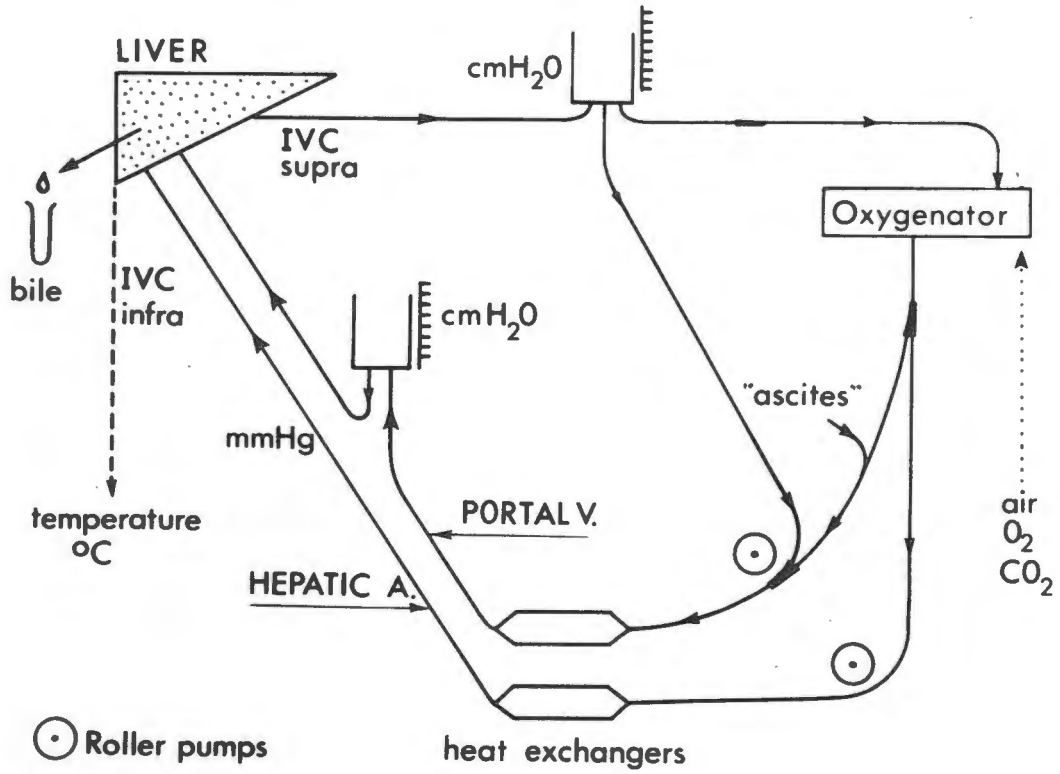
For the pig, the weight of the liver, upon which the flow rate was dependent, was assessed as 3,64g/100g total body weight (live). Since any method of direct weighing involved the estimation of fluid retained within the organ, and cannulae as inserted, it was felt to be as accurate to estimate the organ weight from the total body weight. These weights had been obtained during the transplant programme (119) when the livers from recipients were discarded to the sink and allowed to drain for 15 minutes before weighing. This weight was then related to the total body weight of the animal just after anaesthesia and a mean of fifty estimations was used in the perfusion programme. It has been noted, in justification of the use of this mean, that the pig liver can more than double its empty weight when maximally distended (292). The calf and lamb livers were assessed at 1-2% total body weight (247).

ix) **Technique**

Perfusion was commenced approximately 40 minutes after initial devascularisation of the liver, after pre-perfusion samples had been taken and the Astrup corrected. The priming blood was warmed to 38°C before insertion of the liver into the circuit; some authors prefer to warm blood and liver simultaneously (156), but it was felt that this might give rise to some problems of haemolysis.

Factors considered during initiation of the perfusion were (i) the weight of the liver, and (ii) arbitrary perfusion pressure maxima of 15 cm H_2O in the portal system and 100 mm

Figure 8 : Diagrams of the circuits used for experimental and clinical perfusions.


LIVER
cmH2O
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Oxygenator
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PORTAL V.
temperature
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air
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CO2
HEPATIC A.
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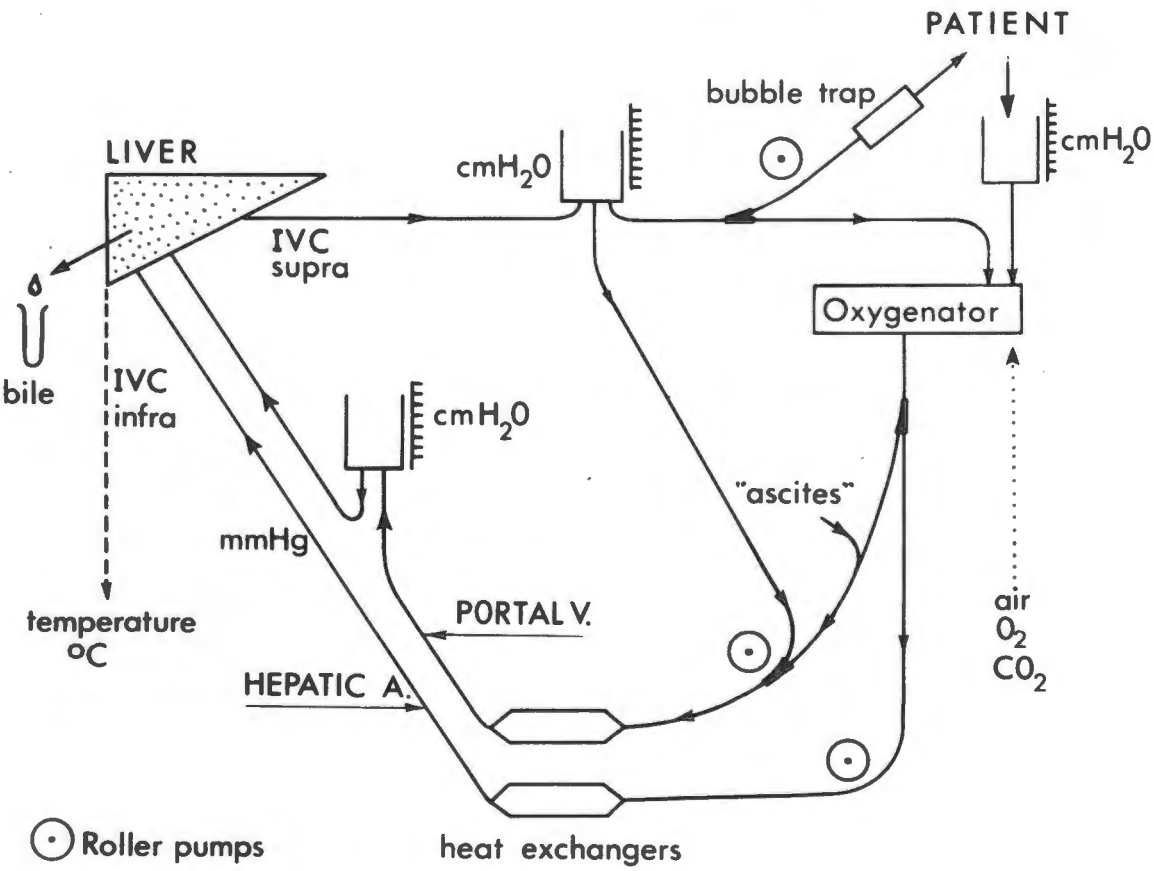
PATIENT
bubble trap
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LIVER
cmH2O
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Oxygenator
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IVC
infra
cmH2O
"ascites"
mmHg
PORTAL V.
temperature
°C
air
O2
CO2
HEPATIC A.
Roller pumps
heat exchangers

Hg in the arterial system. Perfusion was commenced simultaneously through both pumps adjusted to flow rates which resulted in these pressures. Over the course of the first 30-45 minutes, in most cases, the flow rate could be increased gradually as the liver rewarmed and the intravascular resistance declined. It was hoped in all cases to achieve a total flow rate of 1,0 ml/g/min with a distribution of 70-80% flow through the portal vein. Resistance reduced rapidly as the temperature approached 35-36°C, after which rewarming proceeded more slowly. After achieving the estimated total flow, this was not diminished unless gross elevation of pressures occurred. In general, it was preferred to observe subsequent alterations of pressure at the chosen flow rate.

A negative pressure was maintained in the venous return line by positioning of the catheter as detailed previously. Bile samples were collected hourly, and the ascites which flowed from the liver surface and the incised lymphatics was returned to the prime.

In perfusions using the Rygg bag oxygenator especially, an additional line was taken from the venous return reservoir to the portal system to allow the admixture of venous and arterial blood in order to achieve partial saturation of the portal supply. Flow through this line was adjusted by a screw clamp according to the oxygen saturation measurements in the portal blood. This technique was similar to that of Kestens et al (136), but they are the only other perfusion group to use this attempt at a more physiological system.

x) **Flushing of the liver**

Preparation of the liver prior to perfusion is an extremely important stage in the experiment, yet very little study has been made of the subject. The objects of flushing the liver are short term preservation, and the removal of blood elements from the organ. Four features of the procedure will be discussed - the constitution, temperature, volume and pressure of inflow.

(a) Constitution

In most instances, initial rinsing of the liver has been performed with Ringer lactate which had been found satisfactory for preservation of the kidney. However, in a study of the effects of various solutions upon aspects of function of the perfused liver, Abouna (2) has shown clearly that Ringer lactate results in more damage than other solutions. It is suggested that the simple electrolyte solution without colloid osmotic pressure and deficient in energy substrates with a low pH is quite unsuitable (7). From his evaluation, he preferred to use a solution containing high levels of potassium and magnesium, with Dextran, insulin, heparin and procaine. Subsequently Abouna has shown further that a solution with high potassium levels as advocated for long term kidney storage (47) is not effective as a preserving solution for the liver. Indeed, he concludes that while most mammalian cells share common needs and undergo similar transmembrane shifts during hypothermic preservation, there appear to be important differences in

the metabolic requirements and in preservation conditions among different organs. This is an aspect which requires a great deal of further study.

In the study to be described, several different fluids were assessed (Table III). The first was the commercial preparation - TisUsol - which has the advantage of added sulphate and phosphate; the initial pH of this fluid was corrected to 7,4 with the addition of 10 to 15 mEq/l of 4,2% sodium bicarbonate. A second solution which was used was the plasma/bicarbonate/dextrose solution of Schalm (237) with which livers had been successfully preserved for periods up to 10 hours (250). The third solution used was a modified Krebs Ringer Bicarbonate solution with which isolated rat livers had been successfully perfused (58).

(b) Pressure

Little attention has been paid to the pressure under which flushing, especially of the portal system, has been performed, and a remarkably wide range has been reported. In the experiments under review, the bottle containing the flushing solution was hung no higher than 20 cm H_2O above the porta hepatis. The arterial system was flushed with a fluid from a bottle hung 1 metre above the porta hepatis, and intermittent increase in this pressure was achieved by pressure on the "pump" of the intravenous infusion set. During flushing, the venous return line was intermittently clamped to clear the furthest edges of the liver.

(c) Volume

The volume selected for rinsing of the liver should probably be related to the weight of the organ since the object of flushing is the removal of blood. However, usually a standard volume was used which was selected by experience of the appearance of the liver. The organ cannot in fact be completely rid of blood, and flushing was continued until the effluent was clear. Arbitrary volumes used initially were 1,0 litres through the portal vein and 0,25 litres through the artery. Later this was increased to 1,75 litres through the portal vein and 0,25 litres through the artery. In clinical perfusions, this volume was increased to a total of 3 litres, but it cannot be said that the appearance of the liver was different. A wide range of volumes has been used by other workers, with a maximum of 10 litres, and a study of this aspect is much needed, probably with histological control of the flushed livers to assess the presence of red cells after flushing.

(d) Temperature

It is claimed that the liver can withstand only 4 minutes of warm ischaemia, but few studies exist of this fact (256). As was detailed previously, the reports concerning the results of ischaemia to the liver are varied and in most instances it has been preferred to cool the organ before institution of perfusion. Initially, thus the livers were flushed with solution at 4°C. Later, it seemed that a better flush might be achieved if there were no vasospasm as a result of the

Table III : Balanced salt solutions used for flushing and perfusion of the liver as detailed by Abouna, and in this study.

Abouna's solutions

	R/L	I	II	III	IV	V	TisUsol*
Na	131	110	80	130	130	130	137
Cl	111	80	110	112	111	110	142
K	5	14	60	28	5	5	5.8
Ca	4	4	4	4	4	4	-
Mg	-	2	2	2	16	2	1.6
Lact.	29	20	-	-	-	-	-
HCO_3	-	24	30	36	30	25	-
SO_4	-	2	2	6	6	2	1.6
PO_4	-	4	-	-	-	-	1.1
Dextrose	-	200	200	200	200	200	100
Insulin	-	-	-	10	-	-	-
Dextran	-	-	-	-	-	6%	6%

Krebs Ringer bicarbonate: modified

NaCl	9.0 g/1
KCL	1,26 g/1
$CaCl_2$	0,49 g/1
KH_2PO_4	0,84g/1
$NaHCO_3$	0,9 g/1

Schalm solution:

ACD plasma	400ml
5% dextrose	80ml
$NaHCO_3$ 4.2%	20ml
	500ml

McCoys Medium 5A. All quantities per litre of medium.

Aminoacids:

DL-Alanine	26,7mg
L-Arginine HCl	42,1mg
L-Asparagine H_2O	45,0mg
L-Aspartic acid	20,0mg
L-Cysteine HCl.H_2O	35,1mg
L-Glutamine	22,0mg
L-Glutamic acid	22,0mg
Glycine	7,5mg
L-Histidine HCl	20,9mg
Hydroxy-L-Proline	19,6mg
L-Isoleucine	39,3mg
L-Leucine	39,3mg
L-Lysine HCl	36,4mg
DL-Methionine	29,9mg
DL-Phenylalanine	33,0mg
L-Proline	17,2mg
DL-Serine	52.5mg
DL-Threonine	35.7mg
L-Tyrosine	18,1mg
DL-Tryptophan	6,1mg
DL-Valine	35,1mg

Vitamins and Electrolytes:

p-Aminobenzoic acid	1,0mg
Ascorbic acid	0,5mg
Biotin	0,2mg
Calcium pantothenate	0,2mg
Choline chloride	5,0mg
Folic acid	0,2mg
Glutathione	0,5mg
i-Inositol	9,0mg
Nicotinamide	0.5mg
Nicotinic acid	0.5mg
Pyridoxal HCl	0,5mg
Pyridoxine HCl	0,5mg
Riboflavin	0,2mg
Thiamine HCl	0,2mg
Vitamin B12	0,075ug
NaCl	6,46g
KCl	0.40g
$MgSO_4.7H_2O$	0.20g
$NaH_2PO_4.H_2O$	0.14g
Glucose I.	4,00g
$CaCl_2$(anhyd)	0,20g
$NaHCO_3$	2,20g
Phenol Red	2,50mg

cold perfusion, and hence the solution was kept first at room temperature, and then at 38°C just prior to infusion. With this last solution, the liver appeared to rewarm much more rapidly with the re-institution of perfusion, and the overall diffusion was more even.

xi) **Priming solutions and additives**

Initially the circuit was primed with the same solution as was used for flushing viz. TisUsol or Krebs Ringer bicarbonate, but later an attempt was made to improve and sustain function by the use of a tissue culture medium for priming and various additives as constant infusion. The tissue culture medium selected was McCoys 5A (see constitution). Whilst this was used because of the wide range of substances available in it, there is no documented evidence of its value in the perfusion of isolated organs. In addition, it is not known whether the concentrations of amino acids are optimal, especially for the liver. In some rat liver experiments, various alterations in amino acid levels have been shown which are thought to inhibit some aspects of function (198). However, it was noted that with the use of this medium, an improved rate of synthesis of urea occurred, which might be interpreted as an indication of improved function. An additional advantage observed with the use of the medium was that the phenol red included as an indicator of pH was actively excreted in the bile and could perhaps be used as a valuable indicator of biliary secretion and transport.

During the course of various perfusions, several additives were given both by single injection and by constant infusion. Where relevant to a small series of experiments, these will be individually described before the presentation of the experiment, but in several instances, additions were made to a large number of perfusions. In an attempt to provide substrate and stimulus for biliary excretion dehydrocholic acid and secretin were added, and insulin was given for its role in maintenance of the intracellular potassium and glucose levels. These three substances were given in a "cocktail" prepared with arbitrary concentrations - soluble insulin (pork) 100 units, dehydrocholate (Boots, England) 100 mg, and secretin 21 units, - in 100 ml 5% dextrose water to provide a constant infusion of dextrose. This was given over the four hour period of perfusion. (Initially these substances were given as single hourly injections, but on realisation that the insulin particularly would have a half life of a few minutes before destruction in the liver, it was preferred later to give a constant infusion.)

Sodium bicarbonate 4,2% (0,5 mEq/ml) was added arbitrarily at the rate of 1mEq/l per hour. In addition, 3g dextrose per litre of perfusate was added at the commencement of perfusion.

In order to evaluate function, a load of sodium lactate of 3,5g (70% solution B.D.H.) and of ammonium citrate 2g was added before the last hour of perfusion and samples were taken at 5, 15, 30, 45 and 60 minutes thereafter to assess the peak and clearance rate.

When McCoys medium 5A was used as prime, half of the commercial powder required to prepare a litre of solution was dissolved in 250 ml distilled water for the prime whilst the remaining half was dissolved in 50 ml distilled water and was added to the infusion.

As a result of the work of Dawkins et al demonstrating the value of α oxoglutarate in preserving the function of isolated rat livers (58), 15 mM each was added to the flushing solution, the prime and the infusion solution. This was made up in distilled water and the pH corrected to 7,0 with sodium hydroxide.

CHAPTER FOUR

EXPERIMENTS

A - Phase I - control perfusions with pig blood

i) Design
ii) Results
iii) Interpretation

B - Phase II - comparative studies between livers perfused with pig and human blood

i) Design
ii) Results
iii) Interpretation

C - Phase III - clinical application

i) Design
ii) Case Reports
iii) Interpretation

A - PHASE I

i) Design

In order to provide a control basis for experimentation, a series of 23 pig livers was perfused with pig blood for a period of six hours. The purpose of these experiments was to observe the changes in physical and biochemical properties as measured, in order that subsequent modifications could be made to prevent adverse changes.

In this series of experiments

(a) animals were starved for at least 24 hours pre-operatively, and intra-operatively 50g glucose was given in Ringer lactate.

(b) the liver was flushed through the portal vein with 100 ml TisUsol corrected to pH 7,4, and through the hepatic artery with 150 ml 0,9% saline. The temperature of both these solutions was between 10 and 15°C when infused.

(c) the duration of dissection was 60-90 minutes and the liver was removed immediately thereafter with a warm ischaemic interval of 2-3 minutes.

(d) the priming solution was TisUsol plus fresh abattoir pig blood, heparinised and corrected to pH 7,4 with sodium bicarbonate. The final haematocrit was 24%.

(e) partially desaturated blood (70-80% O_2) was circulated through the portal system as described.

(f) the diaphragm was immobile.

The physical characteristics measured included:

The portal, arterial and vena caval pressures, the total flow and percentage portal component and the biliary secretion rate at hourly intervals.

The biochemical investigations made were:

At hourly intervals, the pH, pCO_2, base excess, plasma aspartate transaminase and plasma haemoglobin, lactate/pyruvate ratio and oxygen consumption, plasma osmolality and total protein and albumin. In samples taken pre and post perfusion, sodium, chloride, potassium, urea, glucose plasma alkaline phosphatase and cholesterol.

ii) Results

The results of these experiments are presented in Figures 9-16:

In Figure 9 it appears that the initial portal pressure of 12,2 cm H_2O remained constant for the first three hours and thereafter rose to a final mean of 19 cm. Simultaneously, the hepatic arterial pressure which commenced at a mean of 76 mm Hg also rose at the three hour period

to a mean of 108 mm Hg. At this stage of experimentation, the bile flow was measured according to the number of seconds required for 0,03 ml of bile to be formed, and hence an increase in the number of seconds represents a slowing in the flow rate. Figure 9 shows clearly that there was a considerable reduction in flow rate from the 2½ hour period.

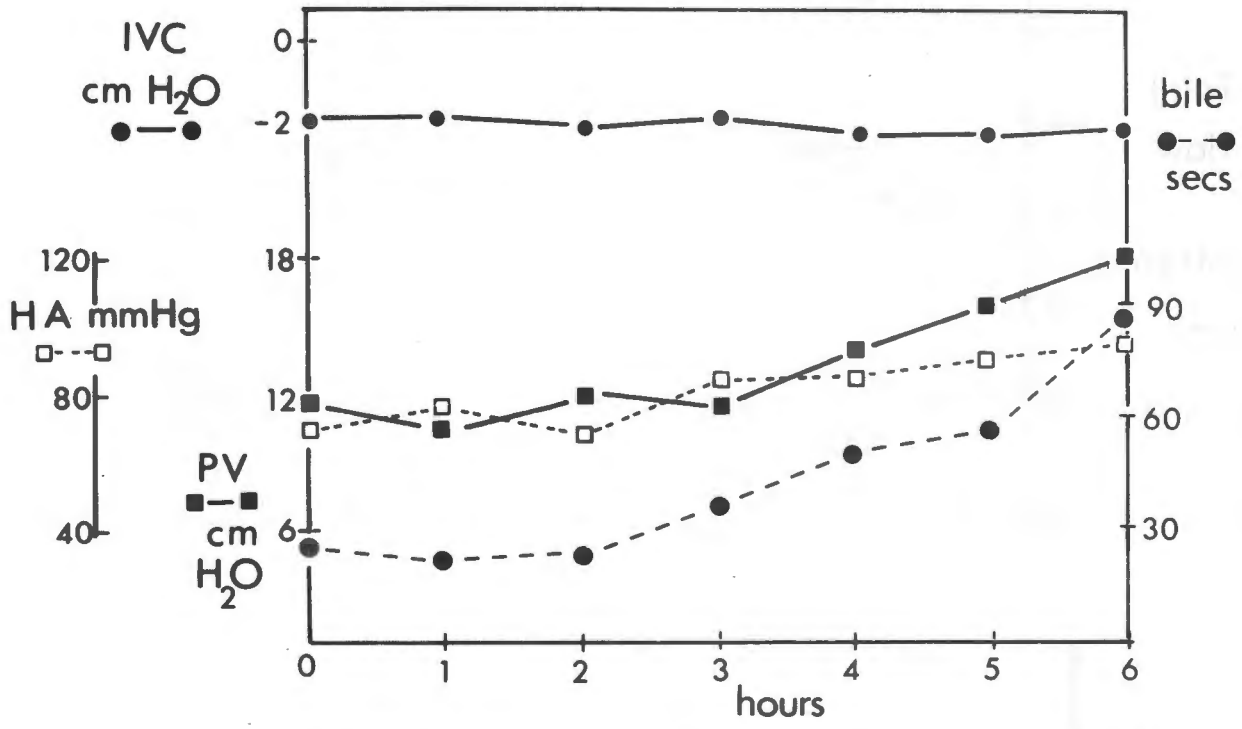


Figure 9 : A graph of the pressures in portal vein, hepatic artery and vena cava, and the bile flow rate.

Figure 10 shows the changes which occurred in pH, which declined initially and thereafter remained within the range 7,40 to 7,43; there was a negligible increase in plasma haemoglobin from 25 to 55 mg/100 ml over the course of perfusion, and in regard to the flow rates, the total flow was increased within the first hour from 0,7 ml/g/min to a final 0,9 ml/g/min which was maintained for the remainder of the perfusion. Of this, the percentage flow through the portal system remained between 83 and 84 for the whole period.

Figure 11 shows the mean values of sodium, potassium and chloride as measured in samples taken pre-perfusion and at 6 hours. There was a significant rise in sodium levels from 152 to 161 mEq/l($p < 0,001$) which was attributed to the addition of sodium bicarbonate to correct the pH. In addition, there may have been some concentration with increase of intracellular water. There was an associated but not significant rise in chloride. Potassium

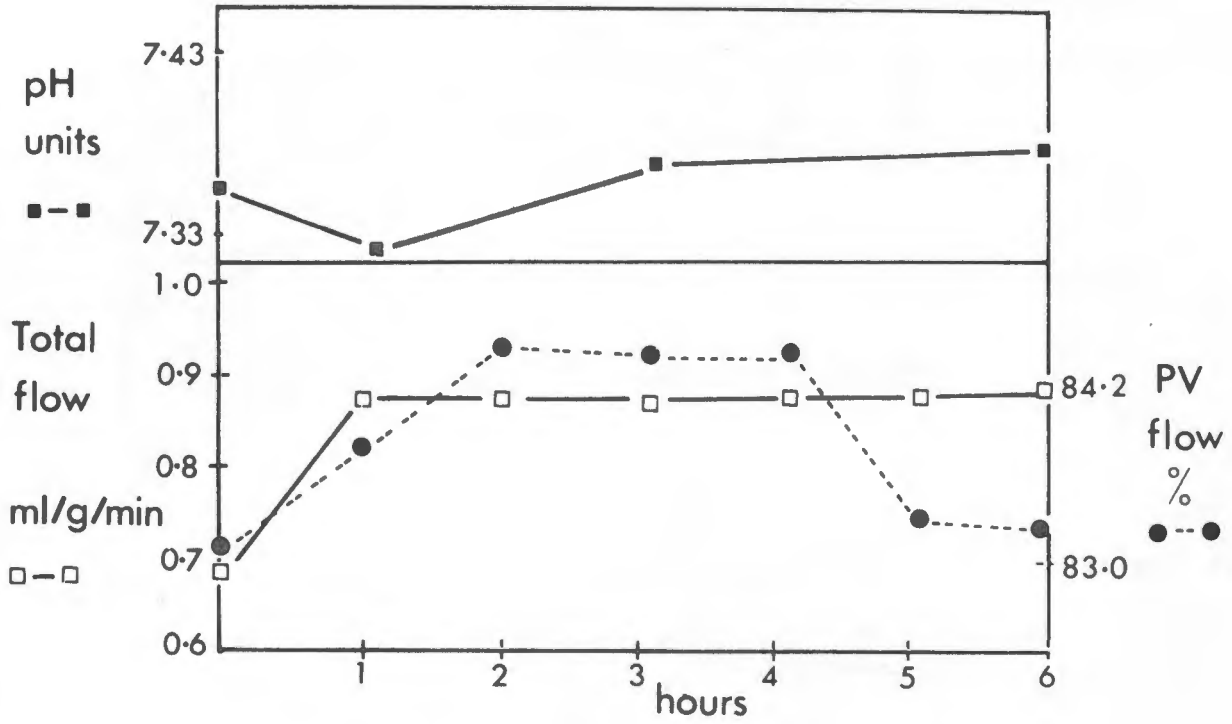


Figure 10 : A graph of the total flow rate with the percentage portal supply, and the pH.

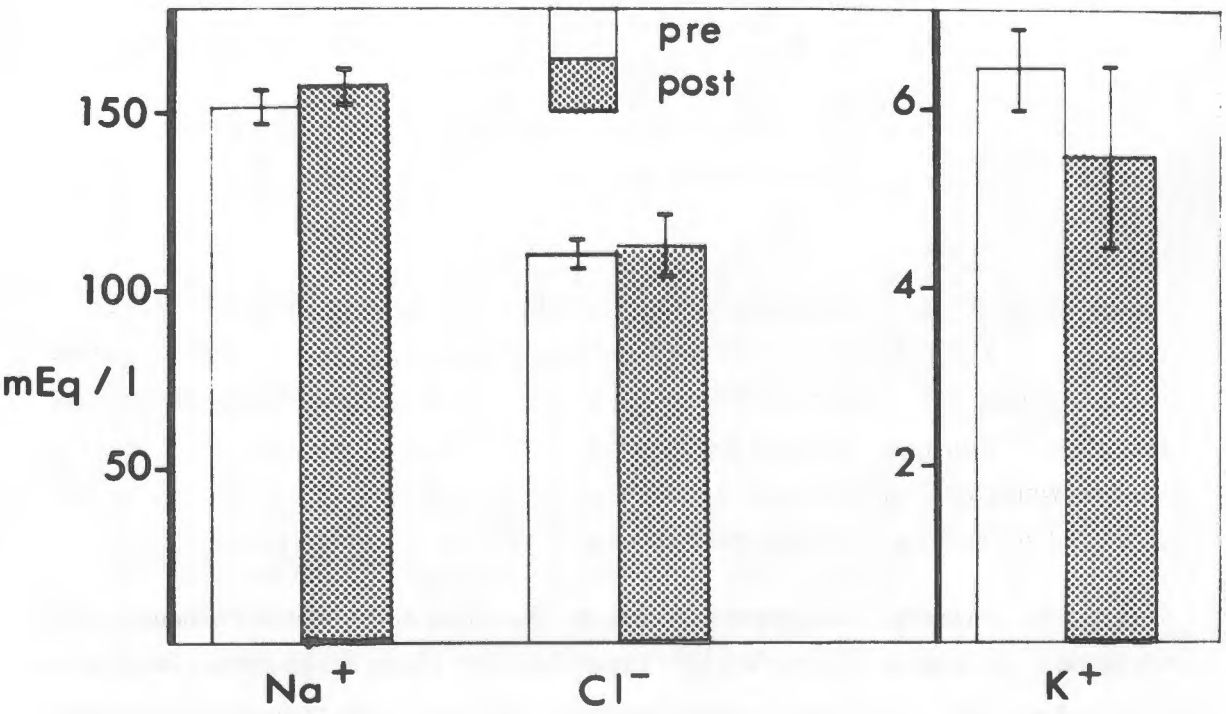


Figure 11 : The electrolyte levels in the plasma before and after perfusion.

decreased from 6,4 to 5,6 mEq/l, and whilst not significant, it was felt that this represented intracellular conservation of potassium.

A mentioned, standardisation of the amount of glucose present in the priming fluid was complicated by differing levels in the donor blood. An amount of 0,56 mol per litre was added but nonetheless wide standard deviations were observed in the mean values as shown in Figure 12. There was a rise in the glucose levels from 7,5 to 9,9 mMol/l which was not significantly different on account of these high standard deviations. However, histological evaluation of pre- and post-perfusion samples showed that there was still glycogen present in the cells after perfusion.

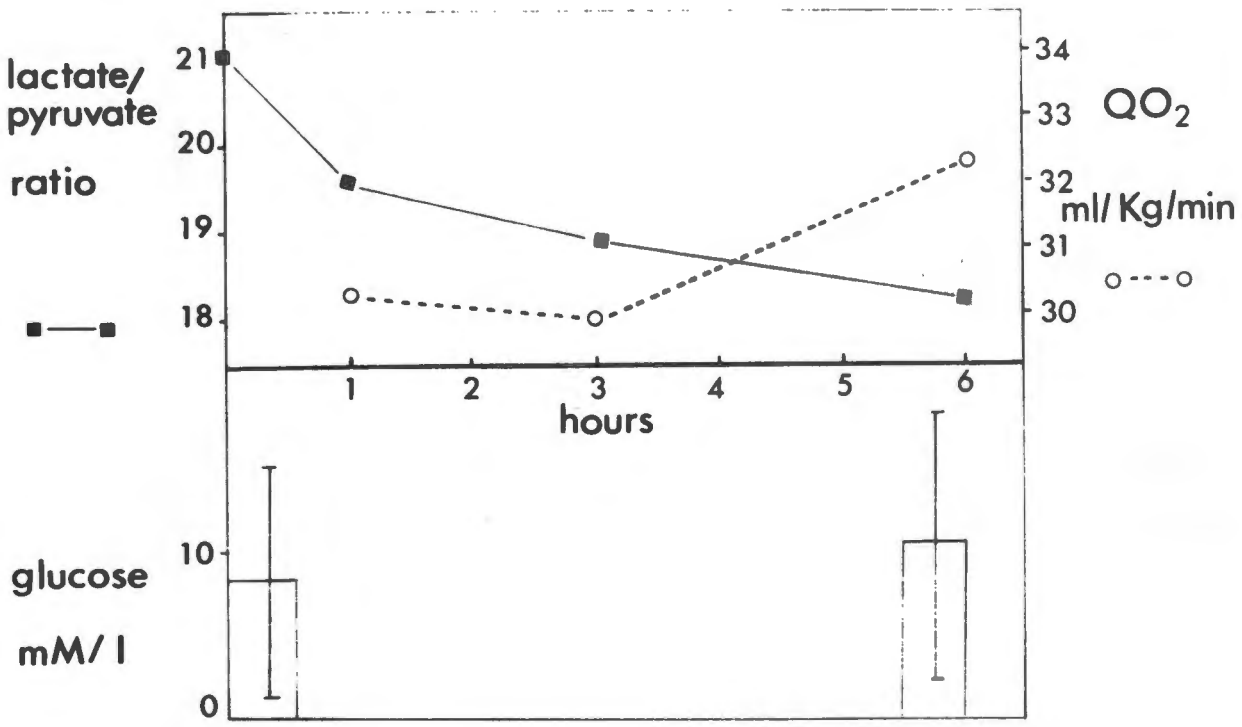


Figure 12 : The glucose levels before and after perfusion, and the lactate/pyruvate ratio and oxygen consumption.

As also shown in Figure 12, the ratio of lactate to pyruvate declined slightly although not significantly. The oxygen consumption was maintained within levels reported to be normal for the isolated perfused liver (3), and the liver in vivo (240), but showed a tendency to increase at the end of perfusion, concurrent with the rise in portal pressure. These combined observations could be interpreted as indicative of a rise in intra-hepatic resistance with reduction in flow rate through the liver.

Figure 13 shows the changes in plasma osmolality and total protein and albumin values during perfusion. The significant rise in osmolality from a mean of 298 to 339 mOsm/l was attributed in part to the increase in electrolytes and glucose as already shown and to urea and other substances which were not measured. The rise might also confirm the concentration which was postulated previously with shift of fluid into the cells. There was no significant difference in protein or albumin levels but the hypoalbuminaemia resultant upon initial haemodilution would certainly have contributed to the development of intracellular oedema.

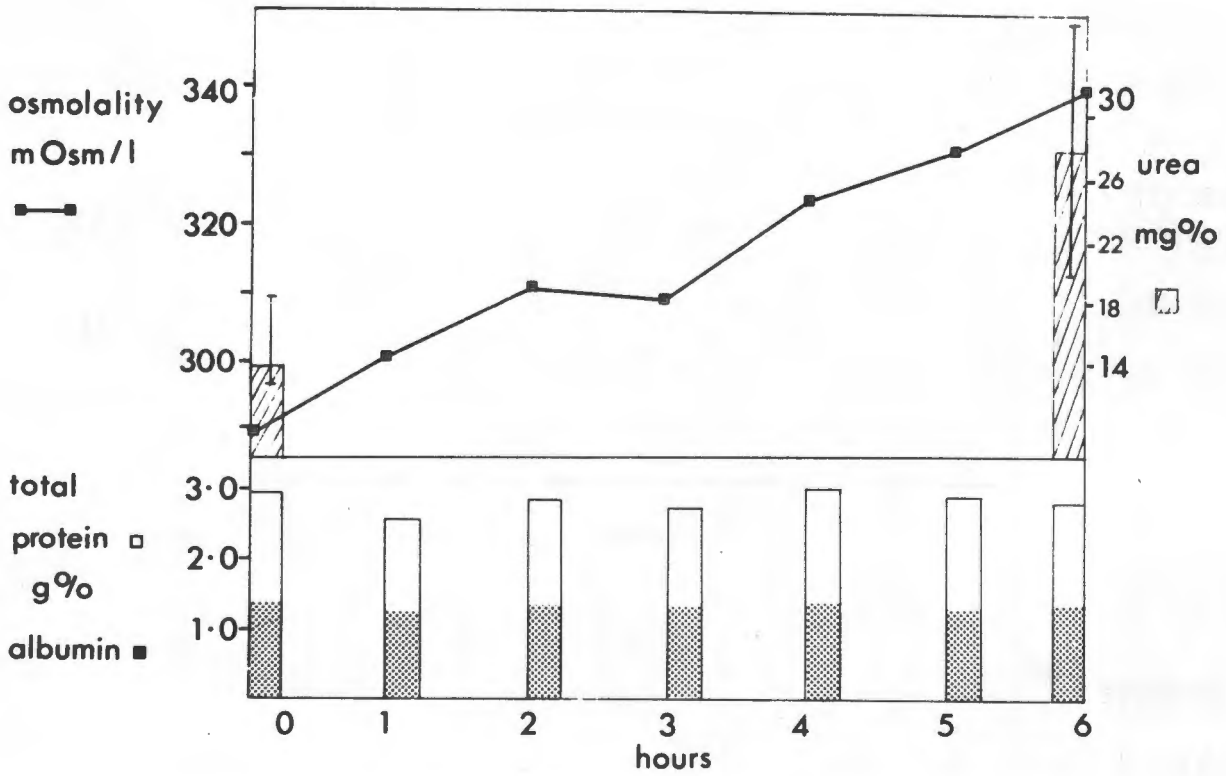


Figure 13 : The plasma osmolality, total protein and albumin and urea levels during perfusion.

Figure 14 shows the acid base status in more detail and shows that the downward trend of pH during the first hour could be attributed to the rise in PCO_2 since the standard bicarbonate remained stable. The slow subsequent rise in pH was probably due to the continued addition of sodium bicarbonate at hourly intervals. However, no marked decline in pH occurred which would have been suggestive of major tissue damage.

Figure 15 shows the changes in plasma aspartate transaminase which was taken as the index of liver damage. Comparison is made with plasma haemoglobin levels over the same

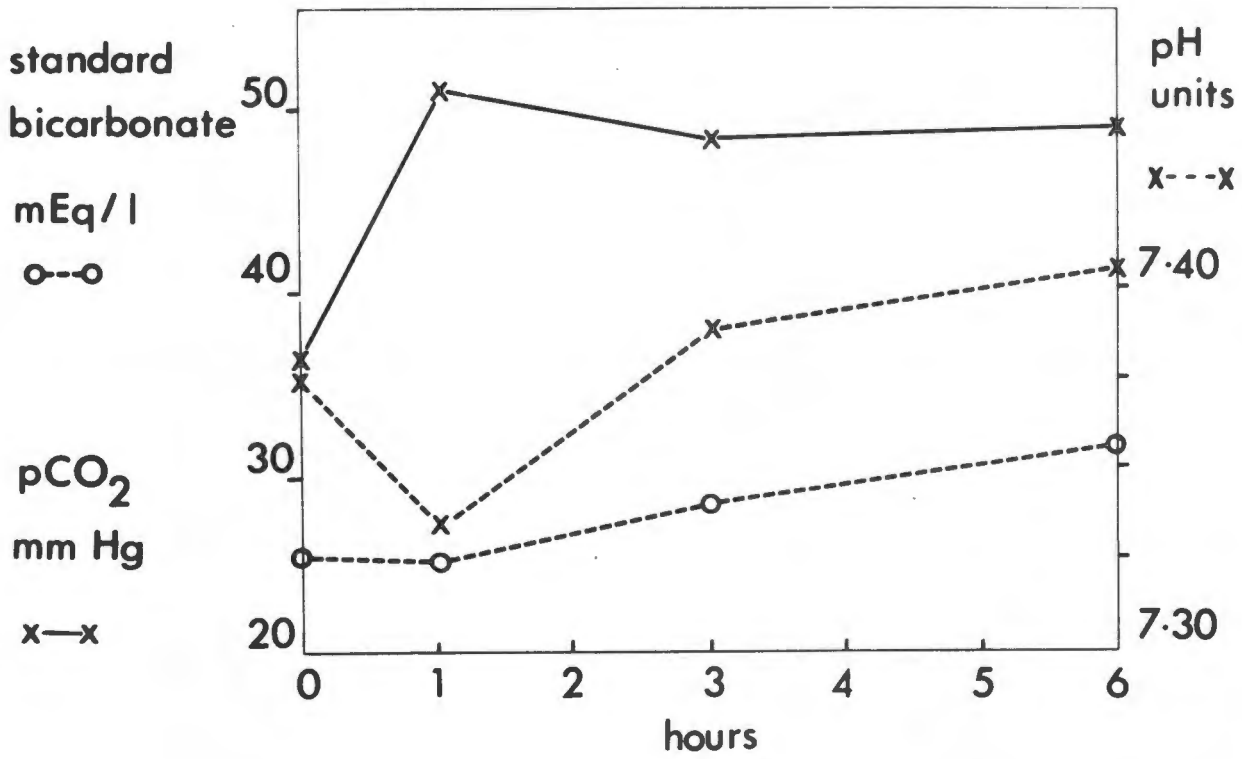


Figure 14 : The acid base balance during perfusion.

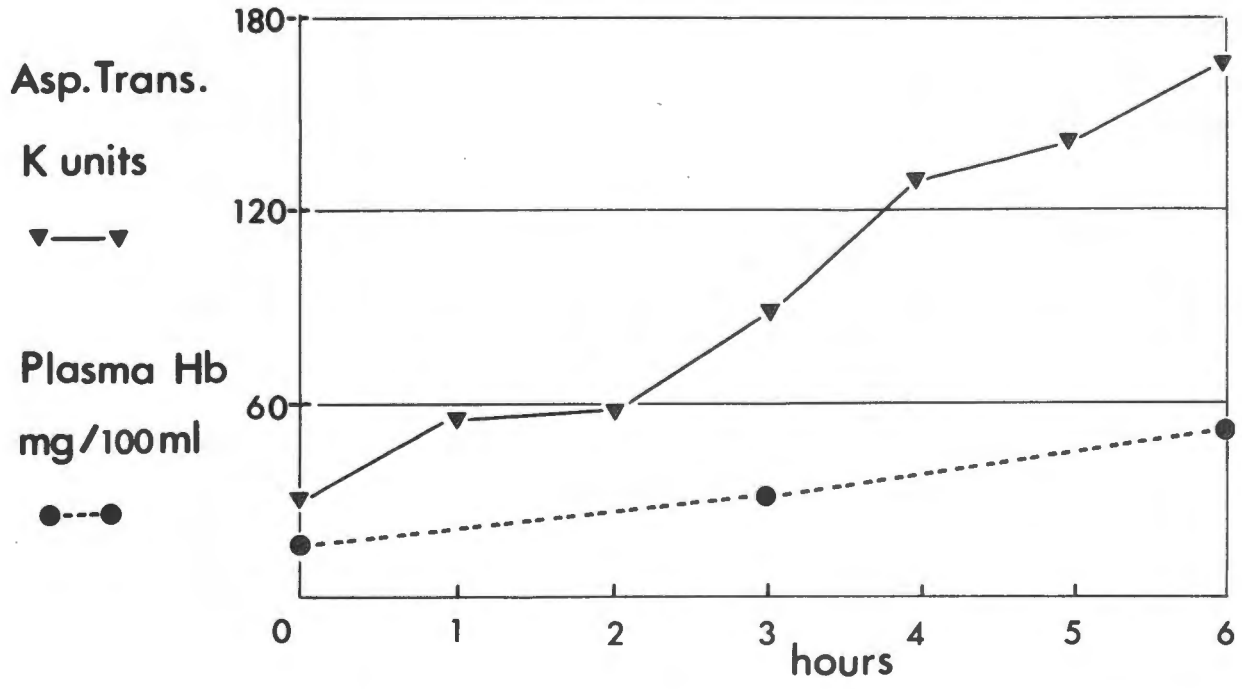


Figure 15 : The levels of aspartate transaminase and plasma haemoglobin during perfusion.

period to indicate that there was negligible damage to erythrocytes and thus little contribution to the levels of aspartate transaminase by enzyme released from erythrocytes. The rise in aspartate transaminase was significant from 32 to 165 Karmen units.

In Figure 16, a further representation of the biliary secretion rate is given, as ml/hour, and in conjunction with the levels of plasma alkaline phosphatase and cholesterol, and biliary osmolality. The minimal decreases in alkaline phosphatase and cholesterol which were not at a significant level could perhaps be attributed to excretion in the bile. The rise in biliary osmolality seemed to parallel that observed in the plasma and probably also represented the increased excretion of sodium in the bile, and concentration of the bile as the flow rate diminished. The observed maximal flow rate was only 10 to 20% of that noted during the operation.

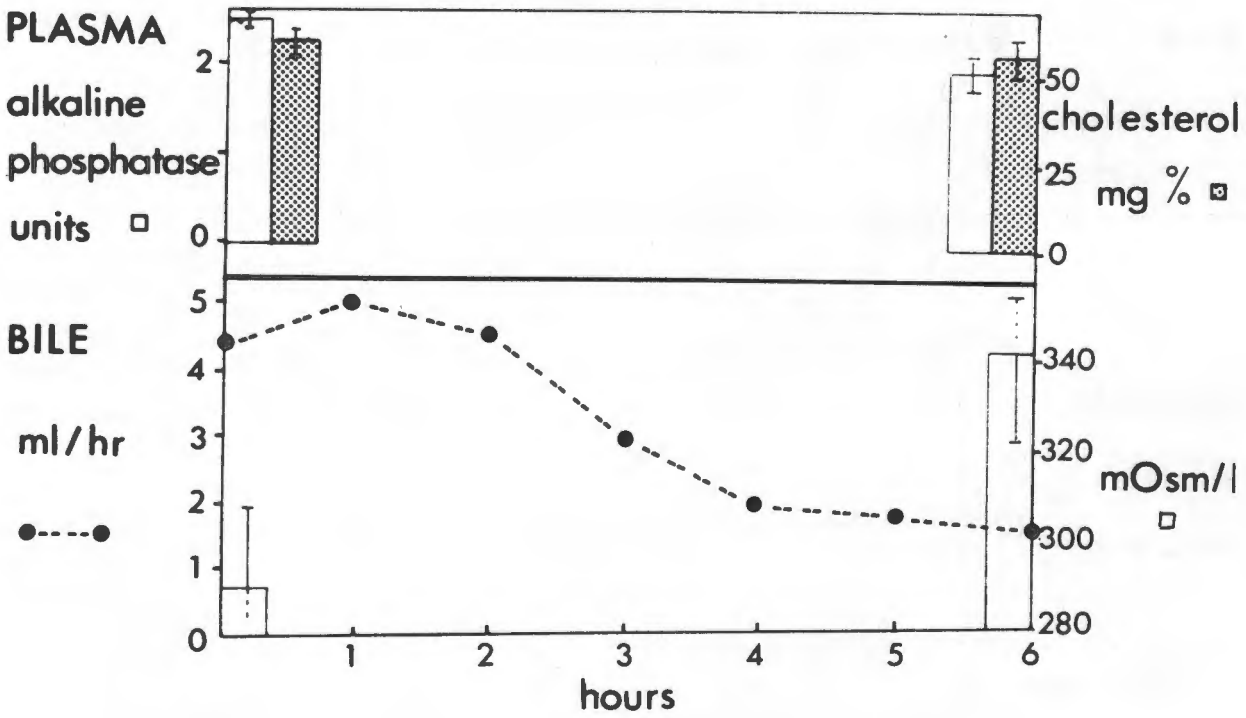


Figure 16 : The biliary flow rate during perfusion, and the plasma alkaline phosphatase and cholesterol levels before and after perfusion.

iii) **Interpretation**

Overall, these results were interpreted as follows:

(a) A stable preparation of perfusion for six hours appeared to have been achieved. The most

striking alterations in the physical characteristics were the slow progressive rise in portal pressure after three hours perfusion, and the rapid decline in biliary excretion rate. A similar rise occurring later in perfusion had been noted by Abouna (5) who attributed it to accumulation in the vasculature of cellular debris resultant upon oxygenator trauma. Such debris was noted in 2 biopsies as described later. The biliary secretion rate was never as rapid as that recorded by others, although the same decline in rate had been previously observed, and no reason for this could be suggested other than an inadequate substrate and hormonal level.

(b) In regard to the biochemical observations, it was thought that the electrolyte levels, especially the constant or slightly declining potassium values suggested maintenance of integrity of the cell membrane ionic pump, and the maintenance of levels of lactate and pyruvate and the acid base status has been interpreted as evidence of metabolism during the period of perfusion. The sustained "normal" levels of oxygen consumption were thought to confirm this, with the reservation of the development of stasis terminally.

The small rise in osmolality which occurred during perfusion suggested that some fluid shifted from the plasma to the intracellular space and a minimal mean increase in the weight of the livers was observed. This was also related to the venous stasis which resulted from inadequate immobile mechanical support of the liver with kinking of the vessels. In addition, a contribution to oedema was probably made by the use of hypoalbuminaemic blood. The slight rise in levels of urea was thought to represent the metabolism of ammonia and other nitrogenous substances, and also perhaps to be the result of gluconeogenesis. The rate would however, also be substrate dependent.

Some evidence of hepatic parenchymal and red cell damage was noted but both compare favourably with other published data (156). In regard to the biliary secretary mechanism, the osmolality appeared to follow the trend of the plasma, and the minimal fall in alkaline phosphatase and cholesterol was attributed to biliary excretion.

B - PHASE II

After the initial evaluation of liver function when perfused with homologous pig blood, a series of experiments was performed to validate the use of such a liver for the clinical hepatic assist.

i) Design

Twenty-one experiments were performed - in 8, pig livers were perfused with fresh heparinised abattoir pig blood; in the remaining 13, 4 pig livers were perfused with human

blood of group O, and 3 each with human blood of groups A, B and AB. Homologous perfusions with pig blood were continued for 6 hours, and heterologous perfusions with human blood were only continued for 4 hours, the period arbitrarily selected for the performance of clinical hepatic assist.

In addition to the features described for phase I, certain modifications were made on the basis of the observations from those experiments.

(a) Human blood was collected on the morning of the experiment, but since this could be obtained only a little later than the abattoir porcine blood, a period of 30 to 60 minutes was allowed following dissection of all livers in phase II.

(b) In an attempt to improve the substrate levels and the level of albumin, the final mean haematocrit was increased to 29%.

(c) The response of the liver to the blood flow offered was monitored by the use of resistance to relate pressure and flow.

(d) Evaluation was made of the capacity of the liver to handle ammonia and lactate, two substances known to be increased in concentration in hepatic coma (25,221). In homologous perfusions, a load of sodium lactate (3,5g) and ammonium citrate (2g) was administered at one and five hours after the commencement of perfusion. Measurement of urea, and lactate/pyruvate ratios was made at quarter hour intervals for the hour after administration of the load. The ability of the liver to return elevated levels to those prior to infusion was measured within the arbitrary period of this hour. In heterologous perfusions, a single load was given three hours after the commencement of perfusion, and clearance was similarly monitored.

(e) Biliary bilirubin levels were measured hourly but were expressed as a total hourly excretion per gram of liver weight to compare with the excretion rate measured in the intact animal during the operation.

(f) Detailed histological assessment of pre- and post-perfusion biopsies was made.

ii) **Results**

Due to the large number of tests performed, only those in which a striking difference was noted will be presented in detail. The remainder are shown in summary in Table VI.

Physical characteristics

Table IV shows that a markedly lesser total flow rate could be achieved through the portal vein in perfusions with group AB blood, than in perfusions with other blood. Table V shows the actual values for mean pressure recordings in the portal vein and hepatic artery for the three

Blood Type	Hours of perfusion						
	0	1	2	3	4	5	6
Group 1							
ml/g/min	0,45	0,84	0,88	0,88	0,88	0,81	0,79
%	82,6	83,8	83,8	83,8	83,5	83,3	83,3
Group 11							
ml/g/min	0,32	0,80	0,80	0,80	0,80		
%	66,0	84,0	87,0	86,0	86,0		
Group 111-A							
ml/g/min	0,40	0,70	0,70	0,80	0,80		
%	82,0	83,0	81,0	83,0	83,0		
Group 111-B							
ml/g/min	0,30	0,80	0,90	0,90	0,90		
%	78,0	84,0	82,0	82,0	82,0		
Group 11-AB							
ml/g/min	0,30	0,50	0,60	0,60	0,60		
%	72,0	71,0	72,0	72,0	72,0		

Table IV : The mean total flow rates and percentage portal flow in perfusions with porcine or human blood.

groups of experiment. The significance of these results is more striking when the individual resistances are calculated.

Resistance - (Figure 17) - In the portal system, not only is there a marked difference in the actual resistances measured, but the levels show a different pattern. In both groups using human blood (II and III), the resistance was very high initially. This tended to decline after the first hour although remaining almost entirely above that of perfusions with pig blood (group I). This pattern was most pronounced when blood of group AB was used. No comparison could be made of resistance after four hours since heterologous perfusions were not continued beyond this time, but the work of Abouna in which a rise occurred after longer periods of perfusion was performed with human blood (5).

Resistance values in the hepatic arterial system showed no particular pattern due probably

to the fact that this blood was pumped under pressure.

A similar pattern of biliary excretion (Figure 18) was seen in all groups as had been noted in perfusions in phase I - viz. a rise in the first hour of perfusion and a gradual decline thereafter.

The results of analysis of serum potassium are shown in Table V. A mean fall of 0,3 mEq/l occurred in group I perfusions with pig blood, whereas a mean rise occurred in all perfusions with human blood - in group II of 2,1 mEq/l, in group III-A of 2,3 mEq/l, group III-B of 0,1 mEq/l and in group III-AB of 3,9 mEq/l. The initial potassium levels in human blood were consistently lower than levels in porcine blood probably related to trauma to erythrocytes during collection of the latter.

Blood Type	Hours of perfusion						
	0	1	2	3	4	5	6
Group 1							
cm H_2O	10	12	13	13	16,5	18	18,5
mm Hg	45	90	105	110	120	130	125
Group 11							
cm H_2O	16	15,5	16,5	16	15,5		
mm Hg	80	60	60	60	70		
Group 111-A							
cm H_2O	14	13	14	13	14		
mm Hg	80	85	85	85	90		
Group 111-B							
cm H_2O	17	15	13	13	13		
mm Hg	65	75	90	90	95		
Group 111-AB							
cm H_2O	16	16	17	16	14		
mm Hg	40	65	75	50	50		

Table V : The mean pressures in the portal and arterial systems in perfusions with porcine and human blood.

Differences in changes in sodium levels were less marked and were related in general to the administration of sodium lactate.

In Figure 19 it may be seen that there was an increase in plasma haemoglobin levels in all perfusions; this was more obvious in the final hours of group III. However, a much more striking picture was observed in comparison of the levels of aspartate transaminase. There was a considerable difference between the levels during homologous and heterologous perfusion, and the difference in perfusions with group AB blood was particularly marked.

An unexpected observation resulted from the measurement of plasma bilirubin (Table VI). In groups I and II, pre- and post-perfusion plasma bilirubin values were less than 0,5 mg/100 ml. In group III, bilirubin increased in both the total and conjugated fractions, the latter forming about two thirds of the total. This increase was greatest in perfusions with group A blood. Estimation of the total bilirubin excreted in the bile over the course of the perfusion, was compared with the excretion in a sample of bile collected during one hour of operation. The perfusion results per hour were expressed as a percentage of the intraoperative collection. In group I, there was a mean excretion of 50%; in group II, 22%; in group III-A, 16%; in group III-B, 55%; and in group III-AB, 28%. No reason can be advanced for these differences except perhaps that the numbers are too small to draw any significant observations. However, it appears interesting that there was the least excretion in group III-A in which the conjugated hyperbilirubinaemia was noted.

Estimation	Group 1	Group 11	Group 111		
			A	B	AB
Sodium mEq/1	29+	24+	10+	9+	8+
Potassium mEq/1	0,3–	2,1+	2,3+	0,1+	3,9+
Chloride mEq/1	3+	2+	4–	4+	4–
Glucose mM/q	3,9+	4,9+	5,2+	2,1+	1,2+
Plasma Osm. mOsm/1	71+	41+	81+	80+	68+
Bile Osm. mOsm/1	72+	30+	70+	42+	42+
Bile sodium mEq/1	6–	11–	4–	6–	4–
Bile potassium mEq/1	1,2–	1,0+	2,2+	3,2+	2,6+
Alkaline phos. units	0,9–	0,3+	1,0+	0,5+	0,4+
Cholesterol mg/100 ml	2,0+	2,0+	17+	7+	15+
Bilirubin mg/100 ml					
total	-	-	4,1+	1,9+	1,8+
conjugated	-	-	3,2+	1,0+	1,1+

Table VI : Mean biochemical observations made during perfusion of the pig liver with porcine or human blood.

Figure 17 : The resistance in the portal systems of livers perfused with porcine or human blood.

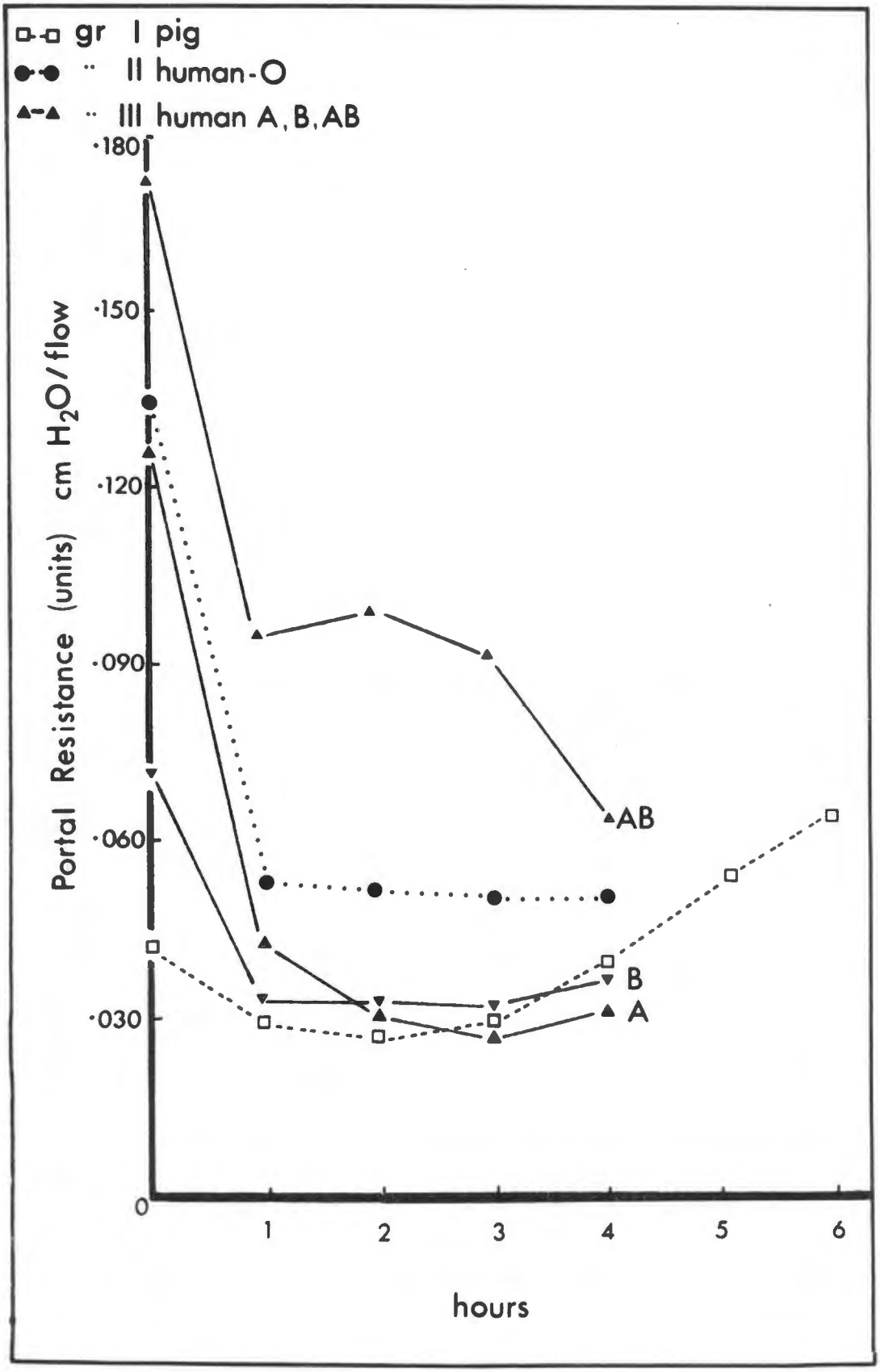

gr I pig
II human-O
III human A, B, AB
Portal Resistance (units) cm H_2O/flow
·180
·150
·120
·090
·060
·030
0
1
2
3
4
5
6
hours
AB
B
A

Figure 18 : The bile flow rates in perfusions with porcine or human blood.

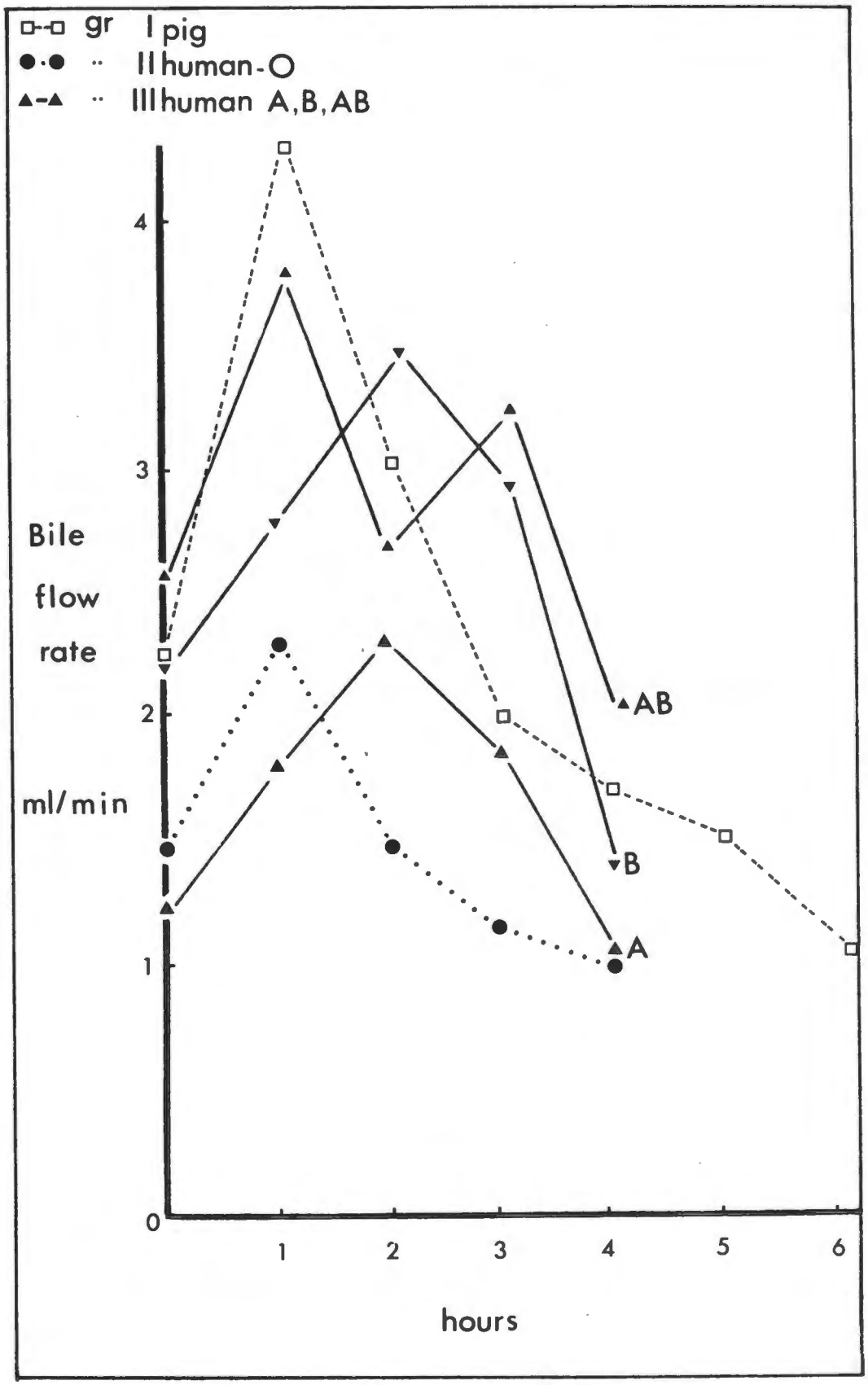

gr I pig
II human-O
III human A,B,AB
Bile
flow
rate
ml/min
0
1
2
3
4
1
2
3
4
5
6
hours
AB
B
A

Figure 19 : The levels of plasma haemoglobin and aspartate transaminase in perfusions with porcine or human blood.

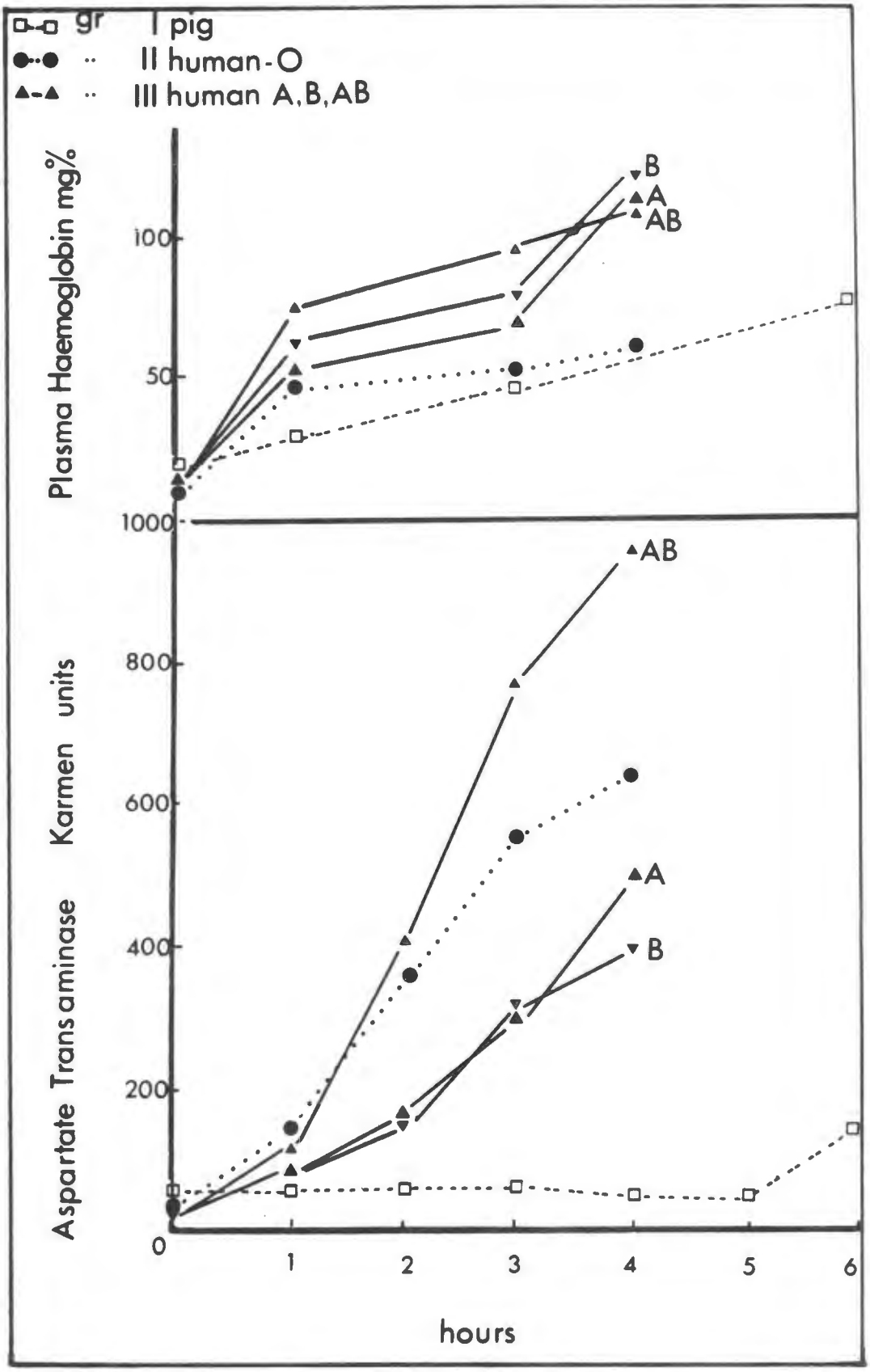
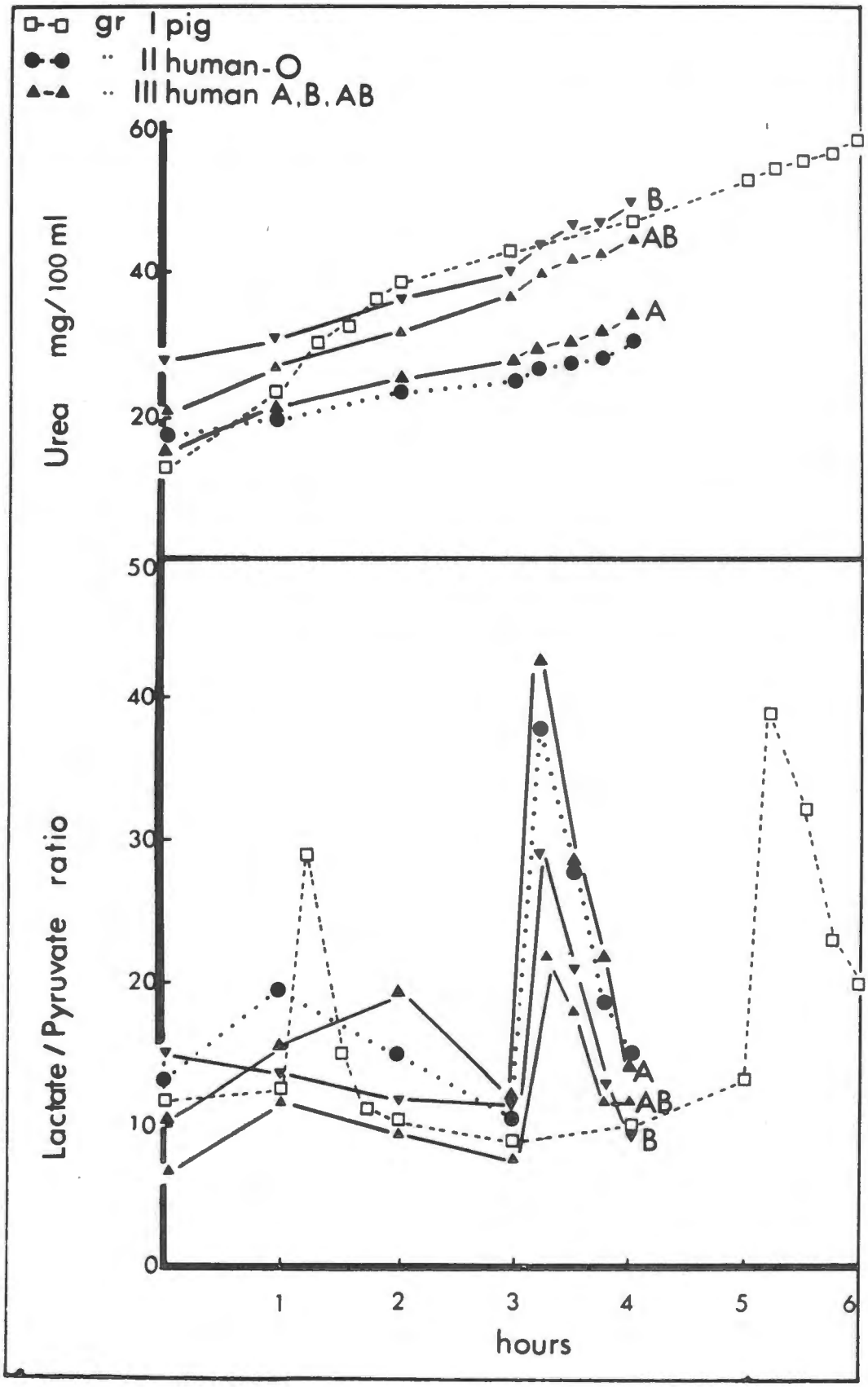

gr I pig
II human-O
III human A,B,AB
Plasma Haemoglobin mg%
100
50
B
A
AB
1000
800
600
400
200
0
Aspartate Transaminase Karmen units
AB
A
B
1
2
3
4
5
6
hours

Figure 20 : The lactate/pyruvate ratios and urea levels during perfusion with porcine or human blood.


gr I pig
II human-O
III human A,B,AB
Urea mg/100 ml
60
40
20
B
AB
A
Lactate / Pyruvate ratio
50
40
30
20
10
0
A
AB
B
1
2
3
4
5
6
hours

Whilst levels of plasma alkaline phosphatase fell during perfusion with porcine blood, there was a slight increase in groups II and III with human blood (see Table VI). A similar pattern was not as obvious when comparing the values for cholesterol, but the actual levels throughout were higher in the human blood. In group I, the biliary potassium fell, but in groups II and III the levels increased during perfusion. This was similar to the pattern seen in the plasma. Biliary sodium levels declined in all groups.

Lactate and ammonia metabolism following a loading dose: (Figure 20).

In most groups the first administered load of sodium lactate was cleared well according to the arbitrary criteria - i.e. to pre-load levels within one hour of administration. Only in group III-AB did the clearance appear slightly less efficient. In group I, the second load of sodium lactate at 5 hours was cleared less completely during the remaining hour of perfusion.

During perfusion with homologous blood, urea increased from 14 mg/100 ml to 54 mg/100 ml; the majority of this increase occurred after the initial addition of ammonium citrate. In perfusions of groups II and III, there was an increase in urea following the infusion of ammonium citrate, which was similar to that following the first addition during homologous perfusion. No significant differences could be detected as the changes are small.

Other analyses (Table Vi)

Plasma glucose levels increased to a variable degree in all groups. There was no marked difference in the levels of oxygen consumption in the three groups, the increases being concurrent with the administration and metabolism of the sodium lactate load. In none of the groups was there a marked alteration in the pH or pCO_2 - in all cases, with the administration of 1 mEq/l/hr of 4,2% sodium bicarbonate, the pH remained above 7,4 units.

The initial mean levels of albumin were higher in the human blood, being 2,4g/100 ml, as compared with 1,6g/100 ml in homologous blood, these levels representing dilution with priming solution to a mean haematocrit of 30%. The total protein and albumin levels altered little throughout the course of perfusions. Electrophoretograms were performed to seek an alteration in gamma globulin levels, but there was no marked difference.

In all groups, the increase in plasma osmolality could be attributed to the addition of sodium in the lactate load, and to the addition of sodium bicarbonate. Increases of bile osmolality probably occurred for the same reason, in addition to stasis and concentration of the bile.

Histology

In biopsies of the livers in group I, a variable degree of sinusoidal congestion and portal tract oedema was seen after perfusion; in three sections, there was evidence of haemorrhage

into the interlobular septa. Biopsis from livers in groups II and III showed similar features of portal tract oedema, but less sinusoidal congestion than in biopsies of group I. In addition, a polymorphonuclear infiltrate was seen which appeared to be migrating from the portal tracts to the sinusoids. In two biopsies from group III, a few intravascular eosinophilic masses were seen, similar to those described by Abouna (5), but these were not wide-spread.

iii) **Interpretation**

This study was undertaken with two objects - (a) to evaluate the nature and degree of damage which resulted from perfusion of the pig liver with human blood and (b) to assess the ability of the liver to metabolise lactate and ammonia, both of which are elevated in hepatic coma.

(a) Evidence of damage

Several factors combined to form a pattern indicating damage to the liver. The initial increase in vascular resistance offered by the portal system to perfusion with heterologous as compared with homologous blood, suggests a vascular component in the response to heterologous perfusion. The effect was less marked in the arterial system, where a pulsatile flow would overcome small alterations in resistance.

In heterologous perfusions, Abouna has shown that resistance increases over the course of perfusion, and has suggested that this is due to the deposition of intravascular debris resulting probably from trauma to blood and parenchymal cells by the pump oxygenator system (5). This has been an occasional finding in this series, but most biopsies showed evidence of stasis which would also contribute towards an increase, particularly in portal pressure. In the homologous perfusions, which continued two hours longer, a slow progressive increase in resistance was noted during the fifth and sixth hours.

When considering the histology, sinusoidal congestion, which was demonstrated particularly in homologous perfusions, could perhaps be attributed to over-perfusion. It has been shown that in the intact liver in situ, there is a variable flow through portions of the hepatic parenchyma at any instant (185,242). Factors which contribute to this are the intermittent negative pressure in the venous return to the vena cava, the diaphragmatic movement and the intra-abdominal pressure. Of these, only the intermittent negative pressure in the venous return is simulated in the perfusion situation. Positioning of the vena caval reservoir results in intermittent filling and emptying of the liver, but no other regulation occurs. The use as a chamber with intermittent positive pressure movement of a diaphragm as described by Abouna (4) or Eiseman (68) might improve this venous stasis.

However, the immobility of the liver and the potential development of stasis and liver damage were similar in perfusions with homologous or heterologous blood and could not alone

be incriminated as the cause of the major liver damage which occurred in the heterologous perfusions.

Evidence for this damage lies principally in the loss of integrity of the cell membrane ionic pump, revealed by the leakage of potassium. There is additional evidence of the release of intracellular constituents in the increase of aspartate transaminase. These increases are only partly related to the increase in plasma haemoglobin which was greater in the last hours of heterologous perfusion.

A marked difference was observed in biliary secretion. In heterologous perfusions, a biochemical profile of biliary obstruction was shown in the slightly slower flow rates, and the elevation of plasma alkaline phosphatase as compared with homologous perfusions. The biliary potassium levels mirrored those in the plasma.

The suggestion of obstruction is confirmed in the heterologous perfusions of group III where a conjugated hyperbilirubinaemia developed over the course of perfusion. This occurred in association with an apparently reduced excretion of bilirubin in the bile. Only in group III-B was there an apparently adequate excretion (when compared with homologous perfusion) whereas in group III-A, impaired excretion of bilirubin in the bile resulted in a marked increase in plasma bilirubin. There was a higher circulating plasma haemaglobin in perfusions of group III which might have provided a load of bilirubin in excess of the transport maximum, especially being heterologous bilirubin. In a subsequent series of experiments utilising human blood with continuous infusion of bile salts and secretin as choleretics, bilirubin levels still rose but in many instances the rise appeared concurrent with the administration of sodium lactate and ammonium citrate. Some competition between the excretion of bilirubin and sodium might be postulated.

(b) Clearance of lactate and ammonia

The metabolism of the administered load of sodium lactate and ammonium citrate shows no marked difference in the rates of clearance in the three groups of perfusion. Clearance appeared to be performed as efficiently in heterologous perfusions at three hours as in homologous perfusions at one hour, although this latter may not be optimal. In addition, the numbers of experiments are too small to draw any but the broadest conclusions.

(c) Other measurements

None of the other biochemical measurements showed any marked difference in the three groups of perfusion. In all cases, plasma glucose levels rose but whether this reflects gluconeogenesis or release of glucose from damaged cells is not clear. There was no obvious difference in the acid base status of the three groups, since the pH could be maintained above 7,4 units with addition of only one mEq/l/hr of sodium bicarbonate. The pCO_2 remained within

physiological limits, while the oxygen consumption commenced at higher levels than has been reported as normal, confirming the stasis which was shown histologically. In all cases, there was an increase in oxygen consumption in relation to the metabolism of lactate and ammonia.

Although albumin levels were higher in the human blood initially, the patterns of protein values and the osmolality of plasma and bile were similar in the three groups. The addition of sodium lactate and bicarbonate probably contributed much to the rise in osmolality.

C - PHASE III

As a result of the observations made in phase II, it was decided to offer clinical hepatic assist to patients in deep hepatic coma, with the following reservations.

i) No relationship between observed function and human blood groups could be noted so no particular selection of the pig could be made.

ii) Despite the initial rise in portal resistance, this appeared to resolve during the first hour of perfusion.

iii) These livers, when perfused with human blood, appeared capable after three hours of clearing lactate and ammonia in a manner similar to that during the first hour of homologous perfusion.

iv) A greater degree of hepatic damage was noted in regard to levels of plasma aspartate transaminase.

v) In interpretation of the biliary secretion rates, plasma alkaline phosphatase and cholesterol, and the appearance of conjugated hyperbilirubinaemia, a biliary stasis related to heterologous perfusion was observed. In clinical hepatic assist, elevated levels of bile acids stored in the patient with liver necrosis, are said to act as cholinergics during assist procedures (4,84), and hence no further secretory stimuli were added. .

vi) It should be borne in mind that this constitutes a heterograft procedure with its attendant hazards.

Hepatic assist by attachment to the pig liver was thus offered to four patients whose clinical courses will be described in detail to allow estimation of the severity of the disease.

Design

For hepatic assist procedures, the preparations of the liver were more prolonged due to the co-ordination of this complex procedure.

i) These procedures were often performed at night when laboratory facilities were sub-optimal.

ii) Livers were flushed with 3 litres TisUsol and 150 ml sodium chloride to attempt to clear most pig blood and protein.

iii) After removal, livers were transported to the hospital submerged in a volume of TisUsol at 4°C. A period of 30 to 45 minutes was allowed for stabilisation of the liver in the circuit before clinical attachment was made.

iv) The condition of these patients was monitored during perfusion by a senior anaesthetist, and by a senior physician, in order that full attention could be devoted to the perfusion.

Case 1:

M.K., an African male of 11 years presented to the Frere Hospital, East London, with mild jaundice 2 weeks before admission to the Groote Schuur Hospital. He was otherwise well and bed-rest was advised. Three days later he was seen to be more jaundiced, and on the same day became drowsy, dizzy and ataxic. He was admitted to the Frere Hospital and exchange transfusion was performed. During the procedure he developed urticaria. There was no improvement in his level of consciousness and for the next 4 days, exchange transfusions were performed at 24-hour intervals. On the 5th day, he answered his name and his level of consciousness was thought to be improving. However, despite further exchange transfusions, no further improvement occurred. After 8 exchange transfusions, he was transferred to Groote Schuur Hospital for further management. On admission he was assessed to be in deep hepatic coma with doll's eye movements and extension of the arms and legs. A sluggish response to pain was observed in the form of decerebrate movements of the limbs. The plantar reflexes were extensor. He was deeply jaundiced and a 2,5 cm liver could be percussed. There were no signs of chronic liver disease.

Preparations were made for pig liver perfusion, but during this time the patient suffered a respiratory arrest which was treated by tracheostomy and intermittent positive pressure respiration with a Bird respirator.

Perfusion was commenced 6 hours after admission and was continued for 3½ hours. There was a flow of 250 to 300 ml/min from the patient into the perfusion circuit and no haemodynamic difficulties were encountered. One hour after the commencement of perfusion, spontaneous respiration resumed, but the assisted ventilation was maintained. Perfusion was discontinued when oozing of fresh blood was noted from the shunt site and the buccal mucosa. A further exchange transfusion was performed immediately after the perfusion in an attempt to remove, at least partially, any heterologous substances transferred from the pig liver. After the perfusion, there was an alteration in the patient's condition in that the reflexes were less brisk and the doll's eye movements could no longer be elicited. The plantars remained extensor. Twenty-four hours later another exchange transfusion was performed, but the plantars

remained extensor and fixed dilated pupils were noted. The patient became hypothermic with a temperature of 95°C. Later he became hypotensive and died following gastro-intestinal haemorrhage.

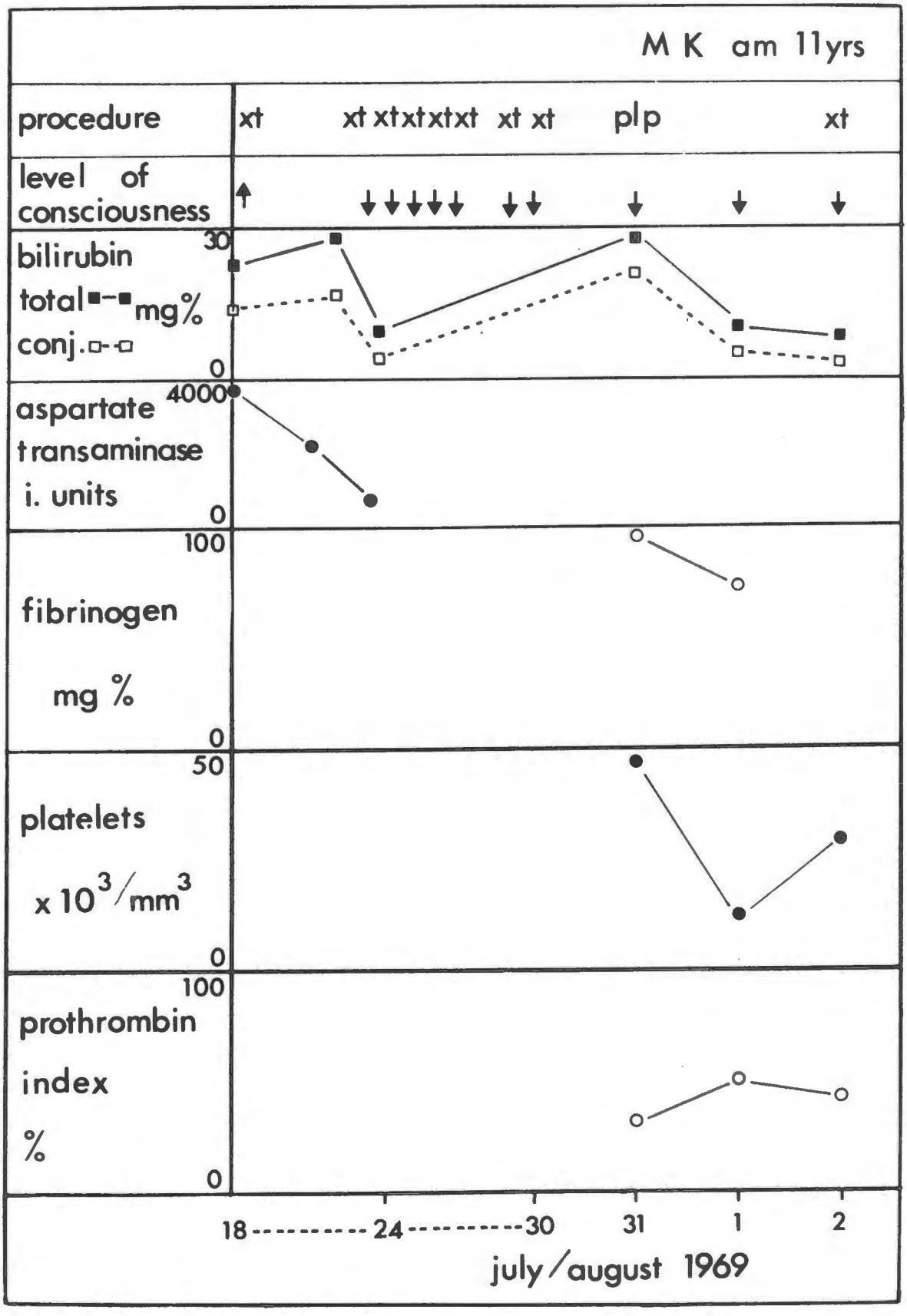
At autopsy the liver weighed 534 grams. There was gross collapse of the periphery of the right lobe and the whole of the left lobe. A small regenerative nodule was noted on the anterior surface. An indistinct lobular area of regeneration in the centre of the right lobe 5-6cm in diameter was noted but not well demarcated. The remainder of the tissue was bile-stained and necrotic. Histology revealed massive necrosis with disappearance of hepatic cells, stromal collapse, infiltration by inflammatory cells and much proliferation of bile ducts. There were surviving islands of regenerative activity in the central zones with ragged vacuolated degenerated cells at the periphery. The kidneys were bile-stained and friable and contained abundant bile stained casts. In the spleen, which was soft and rubbery, there were numerous large monocytic reticulum cells and it was thought that these could be the result of viral infection. Some of the biochemical and haematological findings in this patient are shown in Figure 21.

Because of the danger of exaggerating the haemorrhagic tendency in these patients, heparinisation was kept to a minimum but clumps of cells and strands of fibrin were noted in several of the blood samples and hence heparinisation was increased in the second hepatic assist by the addition of 10mg per hour to the perfusate.

Case 2:

M.A., a Coloured female of 34 years was admitted to the Groote Schuur Hospital with a 3-week history of malaise and lethargy. Four days before admission she noted jaundice and dark urine. Hepatomegaly was detected and she was treated with bed-rest. Two days before admission she became confused. There was a past-history of possible ethanolic excess. On admission, she had asterixis and was assessed to be in light stupor, responding to her name and to painful stimuli. Twelve hours later there was no percussible liver dullness, she was hypertonic and the plantar reflexes were flexor. Later she became deeply comatose and an exchange transfusion was performed. Thereafter, the right plantar became extensor, and reflexes were brisker on the right side. Electroencephalogram showed slowing predominantly on the left side with periodic sharp waves. As there was no improvement in the next 24 hours, a pig liver perfusion was performed. Twenty minutes after the commencement of perfusion, the patient recovered consciousness completely and remained lucid and oriented for 20 minutes. Thereafter, she lapsed back into coma. Perfusion was discontinued after one and a half hours, due to oozing of fresh blood from puncture sites. During the course of the subsequent exchange transfusion, the patient again had a period of lucidity lasting 15 minutes. During the next 2 days, she developed haematemesis and melaena and was treated with two further exchange

Figure 21 : The clinical course of patient M.K.


M K am 11yrs
procedure
xt
xt xt xt xt xt
xt xt
plp
xt
level of consciousness
bilirubin
total ■–■ mg%
conj. □–□
30
0
aspartate transaminase i. units
4000
0
fibrinogen
mg %
100
0
platelets
x 10³/mm³
50
0
prothrombin index
%
100
0
18 ---------- 24 ---------- 30
31
1
2
july / august 1969

transfusions. A picture of consumptive coagulopathy was noted haematologically (see Figure 22). Eight packs of platelet rich plasma were given. Twelve hours later she was awake and talking, and the electroencephalogram showed a marked improvement. During the next two days, she became confused, waves became evident on the EEG and haematemesis continued. An area of liver dullness could be percussed. Despite this, the level of consciousness declined further, the patient became anuric and she died following haematemesis with inhalation. In retrospect, this patient should have had a tracheostomy to prevent this complication.

At autopsy, the liver weighed 905 grams and was soft and pliable. Brightly jaundiced regenerating parenchyma contrasted sharply with deep red areas of necrosis. The regenerating liver was also necrotic and was very soft. The capsular surface was slightly wrinkled suggesting the gross loss of liver tissue which had occurred. Histologically, there was a varied picture with regeneration and centrilobular necrosis with bile plugs and lakes. There was no evidence of pre-existing disease. The stomach contained altered blood and 2 sub-acute ulcers were noted at the cardio-oesophageal junction. The kidneys were oedematous and jaundiced. Some sub-capsular haemorrhage and bile-stained casts were evident. A chromophobe adenoma was noted on section of the pituitary.

Some biochemical and haematological data are shown in Figure 22.

During perfusion of this patient a marked fall occurred in fibrinogen levels and in platelets. However, these were improved by intravenous administration, and it was decided to give both these elements during the course of the next perfusion.

Case 3:

V.M., an African female of 26 years was admitted noisy and unco-operative with a 5-day history of anorexia and vomiting. On admission she was noted to be markedly jaundiced and confused. Both plantar reflexes were extensor but no other abnormal neurological features were noted. A 3-4cm smooth liver was palpated. There were no signs of chronic liver disease. Some 6 hours later she was in deep coma and an exchange transfusion was performed; immediately thereafter a tracheostomy was done. Twelve hours later, she was noted to respond to painful stimuli with decorticate movements of the limbs, and treatment with pig liver perfusion was instituted. This was continued for 2 hours and was stopped due to oozing from the shunt site. An exchange transfusion followed the procedure. The next day, her response to painful stimuli took the form of opisthotonus and another pig liver perfusion was performed. This lasted two hours and was again discontinued because of bleeding. After a subsequent exchange transfusion there was no alteration in her level of consciousness, but a 3-4cm area of liver dullness could be percussed. Another exchange transfusion was performed, and thereafter the patient's level of consciousness began to improve. Over the course of the

next two days, she became fully conscious. However, 5 days later the liver was still impalpable. She lapsed back into coma over one night when she became hypothermic, but she responded to rewarming. During the course of the next 3 days, ascites became evident and melaena developed. She lapsed back into deep coma, possibly due to haemorrhage, and another exchange transfusion improved her level of consciousness to one of mild confusion. She was transfused because of continued gastro-intestinal haemorrhage and the liver edge became just palpable. Two days before death, she developed menorrhagia and required further transfusion. Terminally, she had a massive haematemesis and was thought to have aspirated despite tracheostomy.

At autopsy, there was evidence of pulmonary oedema and inhalation. A florid pneumonia was present and an early lung abscess with associated fungal colonies was seen. Culture yielded a pure growth of Klebsiella pneumoniae. The liver weighed 970 grams and showed three areas of regeneration in the left lobe. The right lobe was flabby, and on histology, there was total necrosis of all liver cells with stromal collapse and aggregation of bile ducts near former portal tracts. There was some evidence of pre-existing fatty change. A bleeding tendency was evidenced by multiple petechial haemorrhages, massive bilateral haemorrhage into the broad ligaments and peri-vesical tissues and a sub-arachnoid haemorrhage over both occipital lobes and part of the upper surface of the cerebellum. The bone marrow showed normal erythrocyte and myeloid series and megakaryocytes.

Some of the biochemical and haematological features of the case are shown in Figure 23.

As a result of the apparently profound effect of perfusion upon the platelet counts, a decision was already in hand to withdraw the procedure until further evaluation could be made in the laboratory. However, with the condition of the fourth patient appearing terminal, she was treated by hepatic assist.

Case 4:

J. v.d. M., a Caucasian female of 17 years had been ill for 5 weeks before admission with dark urine and jaundice. Two days before admission the jaundice deepened, she became stuporose and then lapsed into coma. On arrival in hospital, she was noted to be markedly jaundiced and was making involuntary movements of her limbs of decorticate type. She was in deep coma with decorticate response to painful stimuli. An exchange transfusion was performed six hours later, and there was marginal improvement. A tracheostomy was done and after a 24-hour interval, another exchange transfusion was performed. Although she was again assessed to be slightly improved neurologically, the EEG was isoelectric. Altered blood appeared in the naso-gastric tube. She suffered two generalised convulsions. The next day, while preparations were being made for pig liver perfusion, the patient suffered a respiratory

Figure 22 : The clinical course of patient M.A.

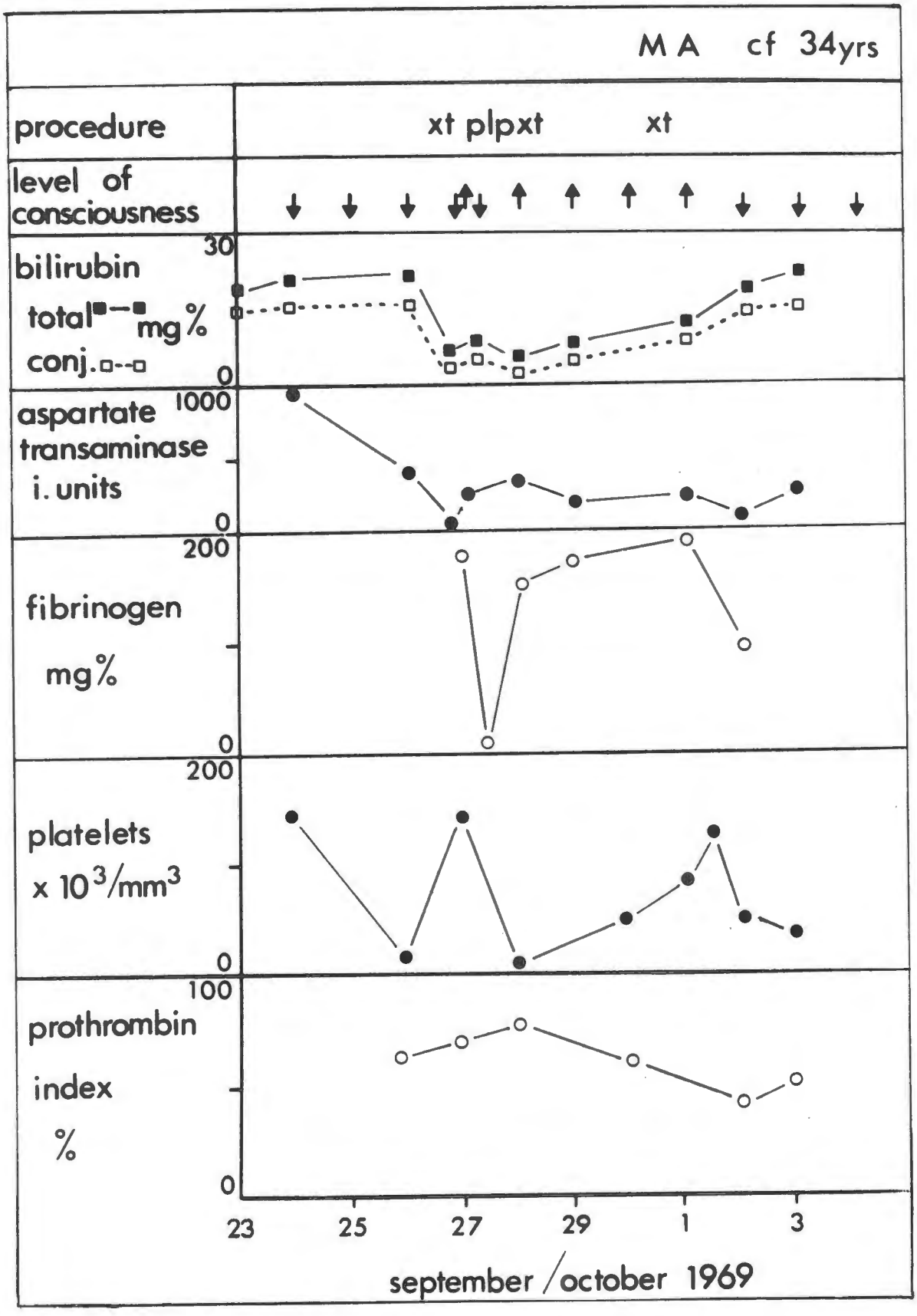

M A cf 34yrs
procedure
xt plpxt xt
level of consciousness
bilirubin
total ■—■ mg%
conj. □--□
30
0
aspartate transaminase i. units
1000
0
fibrinogen
mg%
200
0
platelets
x 10³/mm³
200
0
prothrombin
index
%
100
0
23 25 27 29 1 3
september / october 1969

Figure 23 : The clinical course of patient V.M.

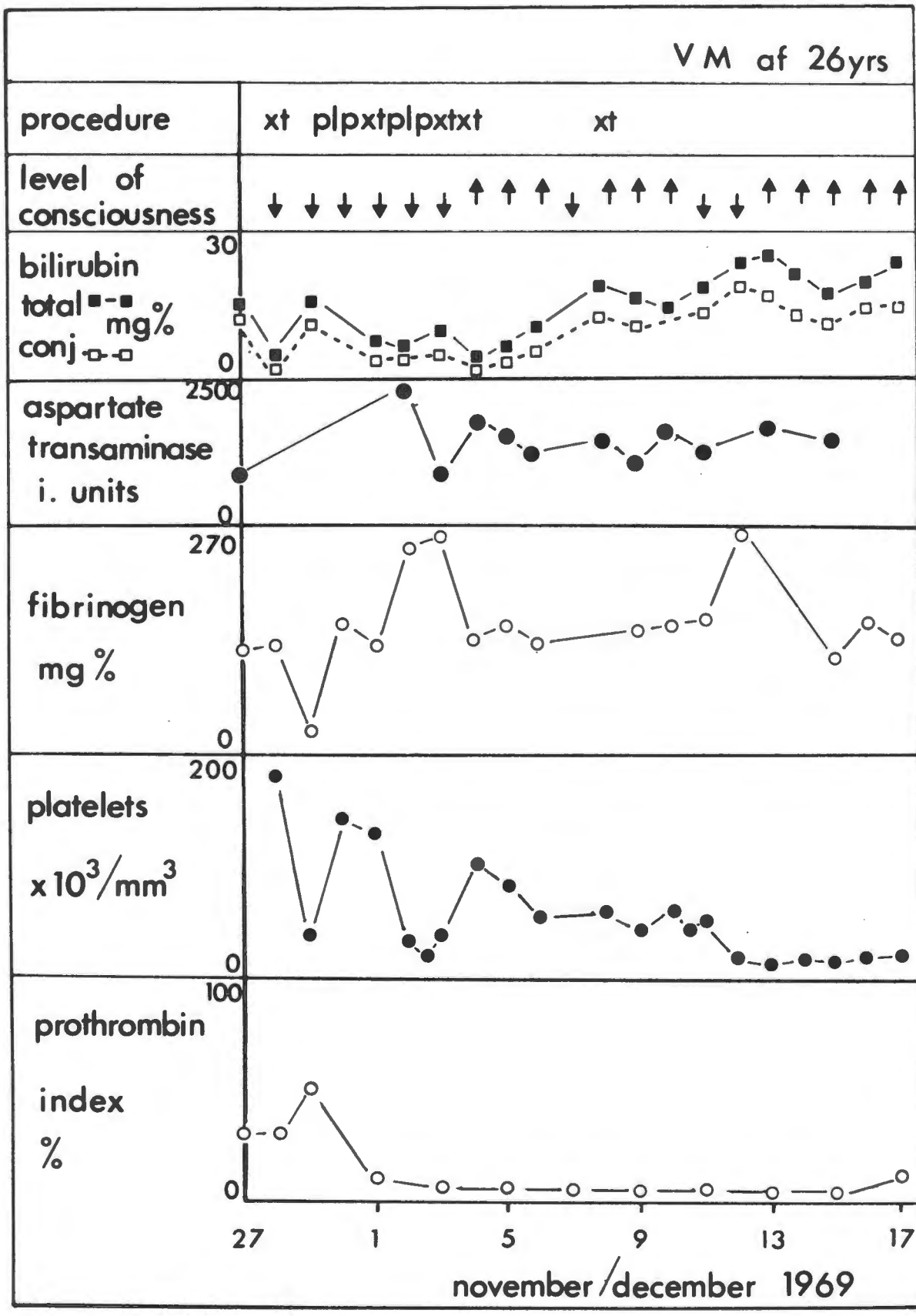
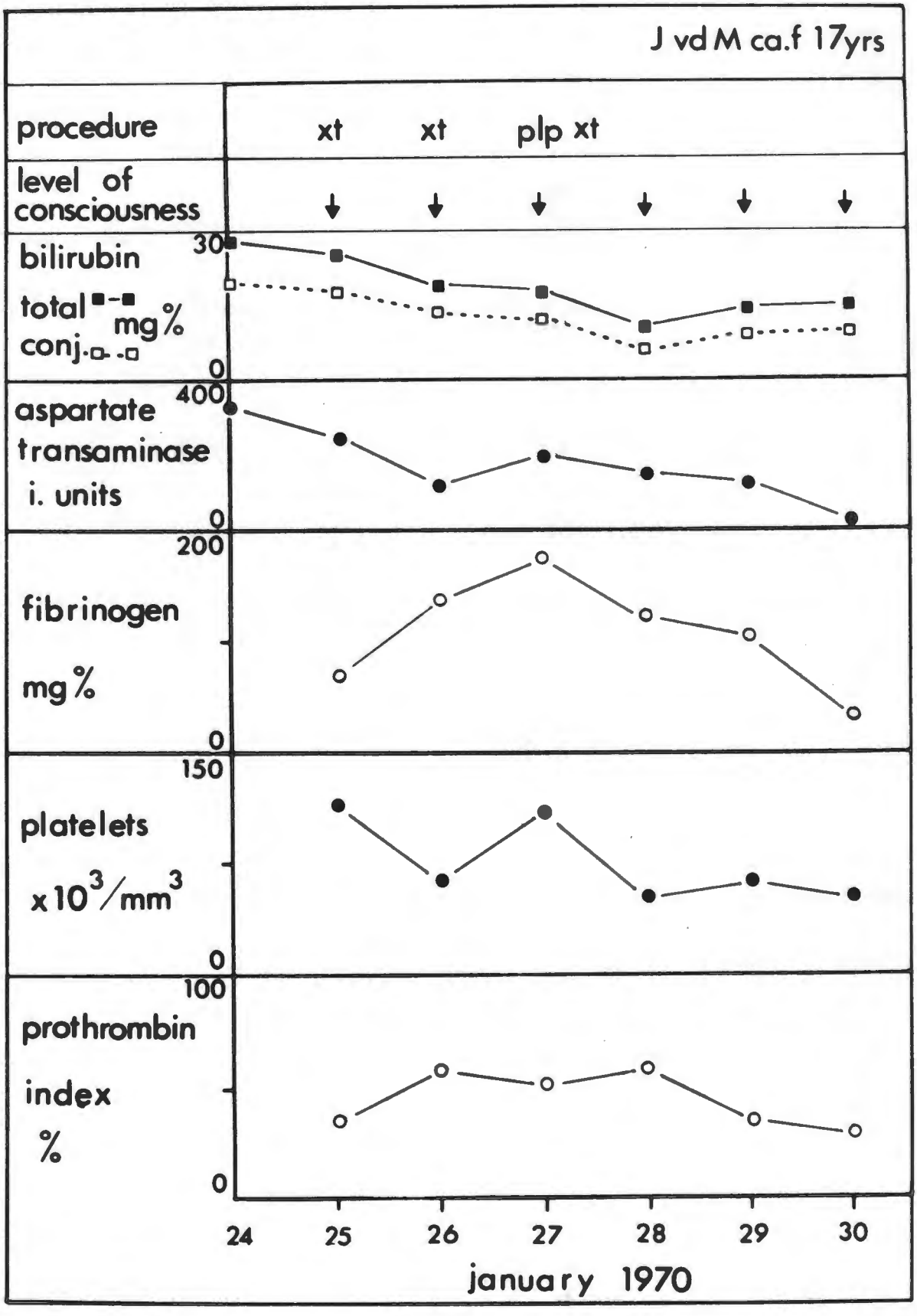

V M af 26yrs
procedure
xt plpxtplpxtxt xt
level of consciousness
bilirubin
total mg%
conj
30
0
aspartate transaminase i. units
2500
0
fibrinogen mg %
270
0
platelets x 10³/mm³
200
0
prothrombin index %
100
0
27
1
5
9
13
17
november/december 1969

Figure 24 : The clinical course of patient J. v.d. M.


J vd M ca.f 17yrs
procedure
xt
xt
plp xt
level of consciousness
bilirubin
total
conj.
mg%
30
0
aspartate transaminase
i. units
400
0
fibrinogen
mg%
200
0
platelets
x10³/mm³
150
0
prothrombin
index
%
100
0
24
25
26
27
28
29
30
january 1970

arrest complicated by cardiac arrest from which she was resuscitated. She became areflexic and suffered another cardiac arrest from which she was again resuscitated. Assisted respiration was commenced. Pig liver perfusion began four hours later and continued for 2 hours being discontinued again because of bleeding from the shunt site. A subsequent exchange transfusion was performed but there was no alteration in the level of consciousness or general condition of the patient. The EEG remained isoelectric and she died 48 hours later.

At autopsy, bile stained effusions were noted in the pleural and peritoneal cavities. The major bronchi were filled with a blood stained mucoid material. The pulmonary parenchyma was oedematous with areas of frank haemorrhage into the lung substance. The liver weighed 999 grams and showed early coarse nodularity. The capsule was wrinkled and thickened. The cut surface showed islands of heavily bile-stained surviving, perhaps regenerating, liver cells with a preponderance of dead rather pale liver stroma. Histology confirmed islands of regenerating cells scattered in a matrix of loose fibrous stroma with a few chronic inflammatory cells. Numerous bile ductules and islands of bile duct epithelium were present in the stroma. Regenerated hepatocytes were represented as eosinophilic "ghost" cells. The spleen was enlarged (weight 423 grams) and the cut surface was dark purple and bulging. There was coffee ground material but no ulceration in the stomach. In the pituitary there was an area of recent infarction. A nodule (0,3cm diameter) of adenomatous hyperplasia of the chromophobe type was seen on section of the pituitary.

The biochemical and haematological data for this case are shown in Figure 24.

Assessment of the livers used for hepatic assist

During perfusion, the livers appeared to function the same as the isolated preparation as described previously. Bile production tended to fall over the course of the perfusions but bilirubin was seen to be excreted in the ascitic fluid exuding from the surface of the liver.

Due to the complexity of the procedure, little monitoring of the liver was performed except for the flow rates and pressure recordings, and the maintenance of acid base balance. Biochemical analyses of the effluent from the liver showed equilibration of the prime with the patient's blood in all cases within the first half hour of link up. No trans-hepatic studies were performed.

Coagulation studies

Figure 25 shows the results in two of the cases (2 and 3) of heparinisation of the patient in addition to the circuit as compared with case 1 where only the circuit was heparinised. In cases 2 and 3 fibrinogen was also given. For comparison, the results in 9 perfusions of the isolated pig liver with human blood are included. There was no apparent fall in fibrinogen levels in

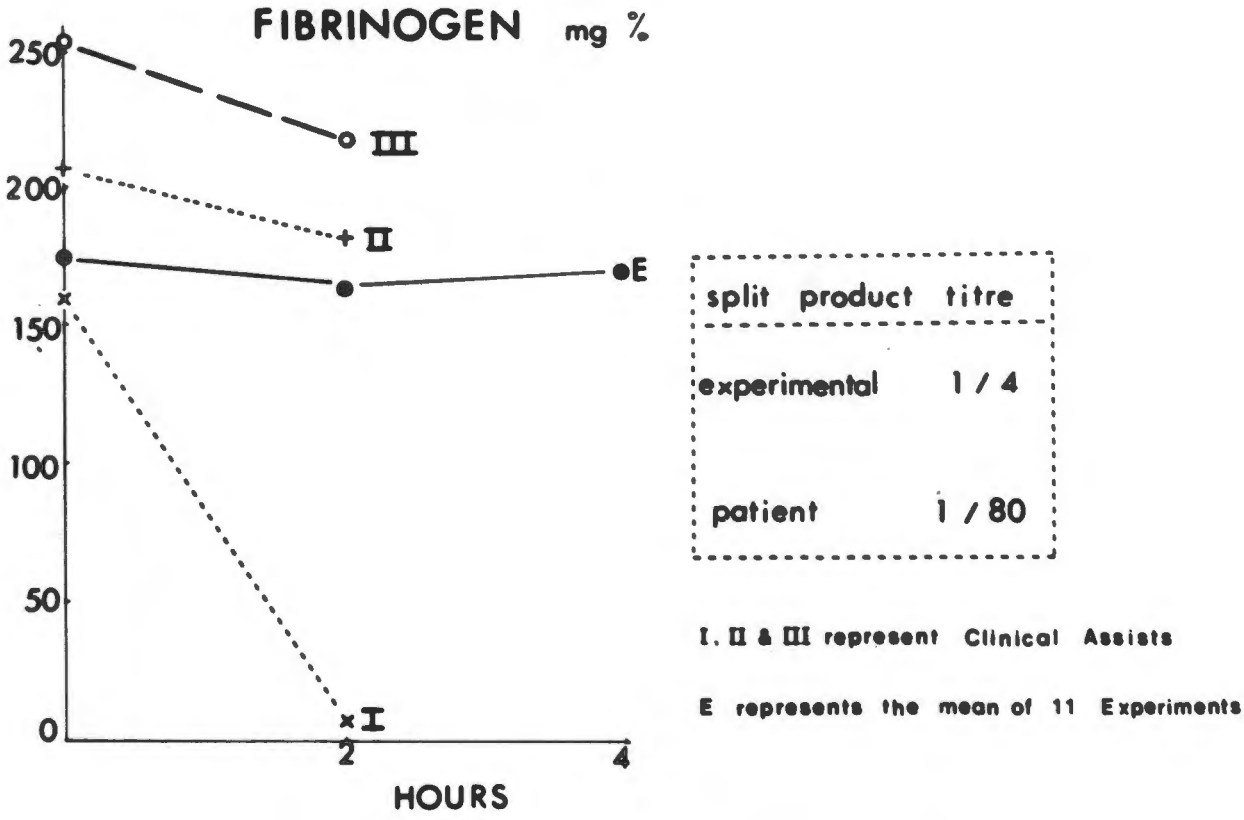


Figure 25 : The levels of fibrinogen and fibrinogen split products during clinical hepatic assist compared with experimental results.

these experiments, nor did split products of fibrinogen appear as they did in the clinical situation.

Although none of these patients survived, the response in improved levels of consciousness in two of the patients which might be related to treatment by the isolated liver, has provided sufficient stimulus to initiate a full investigation of the cause, nature and prevention of the thrombocytopenia which complicated these clinical procedures.

CHAPTER FIVE

THE HYPOTHESIS - that either the circuit or the liver, or a combination of the effects of both, was responsible for the thrombocytopenia which occurred during clinical assist.

A review of the literature:-

a) The circuit

b) The liver - (i) experimental studies

(ii) clinical hepatic assist

(a) - haematological studies

(b) - immunological studies

THE HYPOTHESIS

The hypothesis was proposed that there are at least two possible causes for the disappearance of platelets during clinical perfusion, and as this problem had not been highlighted in other descriptions of hepatic assist procedures, an attempt was made to determine the nature of the complication. It was thought that either the circuit, or the liver could be responsible for the thrombocytopenia, or a combination of both these entities.

a) The Circuit

Trauma to the cellular and protein elements of the blood is known to accompany mechanical pumping procedures (29). The disappearance of platelets is especially related to trauma at the blood-gas interphase of the bubble-oxygenator (15) and damage to leucocytes and erythrocytes has also been noted, with denaturation of protein (293). A great volume of literature has been reviewed in a standard text on heart-lung bypass (88) in order to assess the importance of these changes in patients following cardiac surgery. Whilst thrombocytopenia is noted in such patients, in general it does not constitute a major problem and platelet levels return slowly to normal. However, in the patient submitted to hepatic assist, a number of coagulation defects exist - the failure of the liver to synthesize fibrinogen, and prothrombin (278), and other clotting factors (281), the disordered calcium metabolism (202), and in particular, the spontaneous thrombocytopenia which has been noted in patients with massive necrosis before any form of therapy is undertaken (217,254). This has been ascribed to consumptive coagulopathy and fibrinolysis following major tissue damage. The addition to such a disordered coagulation mechanism of major platelet destruction could well have precipitated the bleeding disorders which were manifest.

b) The Liver

As mentioned in the review of the assessment of function of the liver, a great deal of work has been performed using heterologous blood but in none of these studies has haematological assessment been performed.

(i) In experimental hepatic assist procedures some immunological investigations have been performed, mostly involving the use of the dog. In those experiments in which pigs were used as the divergent species, dogs were noted to collapse with acidosis when connected to a pig liver (98) and other animals were sustained during the procedure, but responded with anaphylactic shock to a subsequent injection of porcine protein. During perfusions, an increase in vascular resistance and decrease in pH were noted (26).

(ii) In clinial procedures a few reports exist in which the results are divergent in regard to the haematological and immunological observations.

(a) Haematological

In only a small percentage of the clinical assist reports is mention made of thrombocytopenia, or allusion made to any bleeding problems (49,202), but in none of these instances was the problem highlighted and in no case was a report made of perfusion termination for this reason. Indeed, in several reports, no mention was made of thrombocytopenia or haemorrhage (50,73,191,211,241). Abouna, especially, has not reported major fall-off even in the long-term multiple species perfusion which was described (6). More recently he has documented 10 patients treated by hepatic assist, of whom 8 suffered acute hepatic necrosis, 5 recovered consciousness and 2 survived (1). He emphasizes the bleeding problems encountered by other workers and attributes his own lack of this complication to the ability of the livers he perfuses to synthesize clotting factors. The synthesis of factors II, V and IX had been reported in a previous series (6). Since an immunological response may be associated with platelet aggregation, and due to the heterograft nature of this procedure evidence was sought of immunological responses in many of the patients reported.

(b) Immunological

In one patient, a temporary hypersensitivity-like reaction occurred with a generalised erythematous rash, diarrhoea, hypotension and vasoconstriction (280). "Considerable bleeding troubles" and thrombocytopenia with increased prothrombin time were noted during and shortly after perfusion. Subsequently antibody activity was detected against the parenchymal cells of human, pig and rat liver, and human kidney (106). However, in view of the major differences of opinion which exist in the interpretation of the presence of such antibodies in many patients with liver disease (213), the significance of these observations is difficult to assess.

In another series of perfusions, it was noted that in the patients' plasma, a decline occurred in α and β globulin, and 19s protein, with a subsequent increase in porcine albumin, $\alpha 1$ and $\alpha 2$ globulin. The ascites exuding from the surface of the livers when perfused represented an ultrafiltrate of the patients' plasma and contained little porcine protein, and in the bile, all protein detected was of human origin (194). In some cases, no porcine protein has been detected immunoelectrophoretically or by immunodiffusion (61), but in others, cytotoxic and anti-pig serum antibodies were noted to develop after perfusion (6).

As a result of this review, it was clearly necessary to perform studies to evaluate the various roles of the circuit and the liver in the thrombocytopenia which had complicated the clinical application of this procedure. In the course of investigation, it was hoped to find a means of preventing this complication.

CHAPTER SIX

A. INVESTIGATION OF THE HYPOTHESIS

Phase IV: Circuit Trauma:

(i) Design
(ii) Results
(iii) Interpretation

Phase V: The role of the Liver:

a — Pig blood + pig liver

(i) Design
(ii) Results
(iii) Interpretation

b — Human blood + pig liver

(i) Design
(ii) Results
(iii) Interpretation

B. THE NATURE OF THE RESPONSE

1. The nature of the heterograft response
2. Platelet aggregation
3. The role of the liver - the reticulo-endothelial system
   - endothelial surfaces and tissue damage
   - coagulation
   - immunosuppression

Phase VI: Is there further confirmatory evidence of the heterograft response?

(i) Design
(ii) Results
(iii) Interpretation

A. INVESTIGATION OF THE HYPOTHESIS

PHASE IV

The effects of the circuit (particularly the oxygenator) upon platelet levels and red cell damage were investigated.

(i) **Design:**

i) Four experiments were performed without a liver, to provide a control series of observations of the platelet counts, plasma haemoglobin and plasma aspartate transaminase.

ii) Four experiments were performed without a liver, with the addition of the detergent Pluronic F68 to the perfusate. This substance had been used experimentally to reduce oxygenator trauma in cardiopulmonary bypass studies on dogs where haemolysis is a major complication (199). In addition it had been used experimentally (55) and industrially as an effective surface-tension reducing agent, and it was thought that this property might prevent the aggregation of platelets which results from direct contact of blood and gas.

iii) Four experiments were performed without a liver in which the oxygenator was first rinsed through with fresh pig plasma in an attempt to cover surfaces which might promote platelet adherence.

iv) In four experiments, the low molecular weight plasma expander, Rheomacrodex was added to the perfusate in arbitrary clinical doses. This substance reduced sludging of blood (120) and it was used in an attempt to prevent platelet aggregation (161).

v) Platelet aggregation has been noted to be associated with the removal of ATP (30) and hence this substance was added to the perfusate in three experiments without a liver

vi) In all the above experiments, the Rygg Kyvsgaard oxygenator was used.

vii) Since none of these additives appeared to be effective, except for the Pluronic F68 of which no clinical application could be found in the literature, a different oxygenator was used. It was not felt justified to embark upon use of a disc-type, especially as the improvement in blood trauma resulting from use of this apparatus is not highly significant (18). Membrane oxygenators are reputed to cause less trauma to all blood elements especially over prolonged periods and have been used to sustain completely the oxygenation of small animals for several days (142). These oxygenators were not available.

A single experiment was performed with the Temptrol oxygenator which combines a heat-exchanging apparatus with the oxygenator. A complication in the use of this

type of oxygenator was the larger priming volume required as a result of the increased area occupied by the heat exchanger.

viii) Finally, the Travenol miniprime 1 LF infant oxygenator was selected because of its lesser priming volume of 1,5 litres which required a slower gas flow to achieve adequate oxygenation. The reduction in gas flow should be associated with less blood trauma. Four experiments were performed without a liver but using this type of oxygenator.

ix) Platelets were counted in a haemocytometer using ammonium oxalate as diluent, as described by Brecher (32).

x) All non-silicone rubber portions of the circuit were re-siliconised at the commencement of each group of experiments.

(ii) **Results:**

Table VII shows the mean results and standard deviations from each of the experiments described above. It may be seen that within the first hour in control group i, there was a fall in platelets to 50% of original levels, but that thereafter there was a less rapid decline over the remaining 3 hours. No improvement was noted with the use of plasma, Rheomacrodiex, or ATP, in fact these levels appeared to be lower, but these differences are not statistically significant.

In the single experiment with the Temptrol oxygenator, the rate of decline was a little slower than in the control experiments.

The use of the Travenol bag in the circuit alone resulted in an improved terminal platelet count. There was virtually no fall in the platelet level within the first hour, and thereafter a decline was noted at the rate of 10% per hour.

The use of Pluronic F68 resulted in a marked improvement in the platelet fall-off, there being only a 10% decrease in the first hour and a final fall of only 30% over the four hour period. This remained an interesting observation only, because the nature of the effect on platelets and blood elements is not defined and no clinical experience has been reported. It was felt unjustifiable to use this substance without some previous clinical use, especially in view of the possibility that the platelets which had not aggregated in the perfusion circuit, might show altered properties when returned to the patient - either with a continued failure to aggregate, or a rebound leading to thrombosis after the drug had been excreted. Whilst it was known that the drug could be measured in the urine (112), neither the route of excretion nor the role of the liver in its metabolism were known.

A lesser rise in plasma haemoglobin and aspartate transaminase in experiments using Pluronic F68 and the Travenol bag confirmed the reduction in trauma to erythrocytes. In addi-

Table VII : Mean values (with±1S.D.) for platelets, plasma haemoglobin and aspartate transaminase in perfusions performed to evaluate the circuit trauma and its prevention.

		Control	Plasma	Pluronic	Rheomacro-dex	A.T.P.	Temptrol	Trav. Bag	Trav. Bag + liver
PLATELETS	0	86 ± 11	74 ± 19	106 + 20	88 ± 9	86 ± 30	101	114 ± 13	86 ± 17
	¼	60 ± 9	66 ± 16	98 ± 25	64 ± 11	83 ± 31	87	104 ± 18	
	½	55 ± 2	58 ± 15	93 ± 26	60 ± 12	78 ± 32	68	106 ± 25	77 ± 11
	¾	46 ± 4	46 ± 9	92 ± 22	46 ± 14	69 ± 27	71	101 ± 16	
	1	45 ± 3	42 ± 9	90 ± 28	39 ± 9	65 ± 31	78	94 ± 24	71 ± 15
	2	44 ± 6	33 ± 5	82 ± 11	30 ± 3	52 ± 24	70	86 ± 17	66 ± 12
	3	46 ± 5	30 ± 6	79 ± 18	24 ± 3	39 ± 19	57	75 ± 22	61 ± 13
	4	39 ± 7	23 ± 7	71 ± 5	21 ± 4	32 ± 15	45	64 ± 15	52 ± 11
Plasma Hb	0	14 ± 5	12 ± 2	9 ± 2	8 ± 4	10 ± 3	24	8 ± 3	12 ± 3
(mg/100 ml)	1	17 ± 7	15 ± 3	12 ± 3	10 ± 2	16 ± 6	29	13 ± 5	15 ± 6
	2	21 ± 10	23 ± 5	15 ± 4	15 ± 2	22 ± 10	34	18 ± 11	19 ± 9
	3	25 ± 15	26 ± 2	17 ± 4	19 ± 2	32 ± 16	32	24 ± 10	23 ± 8
	4	31 ± 6	37 ± 10	21 ± 4	24 ± 2	47 ± 15	45	30 ± 20	57 ± 50
Aspartate	0	58 ± 26	76 ± 18	39 ± 7	33 ± 10	125 ± 106	20	38 ± 12	90 ± 99
Transaminase	1	68 ± 32	84 ± 19	41 ± 8	35 ± 9	67 ± 47	30	38 ± 16	116 ± 96
(Karmen Units)	2	76 ± 40	88 ± 18	42 ± 5	40 ± 10	70 ± 40	29	39 ± 15	126 ± 27
	3	79 ± 44	86 ± 11	41 ± 7	40 ± 10	53 ± 3	34	43 ± 13	154 ± 74
	4	84 ± 41	89 ± 15	41 ± 7	43 ± 8	75 ± 35	32	41 ± 14	161 ± 44

tion, Rheomacrodex seemed to prevent damage to these elements although no effect on platelets was noted.

(iii) **Interpretation:**

The conclusion drawn from these experiments was that the Travenol oxygenator proved superior in regard to platelet damage in the circuit alone.

PHASE Va:

The effect of the addition of the liver to the circuit was then investigated.

(i) **Design:**

In 5 experiments, the pig liver was prepared as has been previously described, and was perfused using the Travenol oxygenator. Platelet counts were performed half an hour after commencement of perfusion and at hourly intervals thereafter.

(ii) **Results:**

It may be seen from Table VII that there was only a slightly greater fall-off in platelets when the liver was added into the circuit. The final count after 4 hours' perfusion was 52% of the original, and there was no significant difference in the other factors recorded. Assessment of the function of the liver as previously described showed no difference with the use of the Travenol oxygenator.

(iii) **Interpretation:**

It appeared that the trauma resultant upon oxygenation might have contributed considerably to the clinical problem of thrombocytopenia.

Thus a series of experiments was begun to demonstrate that the effect of reducing oxygenator trauma was sustained during perfusions with human blood.

PHASE Vb:

In the first experiment using human blood to perfuse a pig liver under identical conditions to those described, very few platelets could be demonstrated in the first half hour sample taken, and no increase occurred during the further course of perfusion. In view of this observation, it was necessary to perform a series of experiments with human blood in which particular attention was directed to the first half hour of perfusion, and to the role of the liver by measurement of trans-hepatic differences in platelet counts.

(i) **Design:**

i) Six pig livers were perfused with human blood for one hour. No attempt was made to match pig and human blood groups and the human blood was used at random as sup-

plied by the Transfusion service; by chance, all blood used was of group A.

ii) Platelets were counted in the perfusate before commencement of perfusion and thereafter at one, five, fifteen and thirty minutes. These samples were taken from the portal reservoir which was representative of the total inflow, and from the venous return which represented the post-hepatic sample.

iii) In order to improve the biliary flow rate which had been found inadequate during the previous perfusions with human blood, the "cocktail" of insulin, secretin and decholin was added by continuous infusion throughout the perfusion.

iv) In three control experiments, pig livers were perfused with pig blood for comparison of the fall-off in platelet counts during the first half hour, and the effect of addition of the "cocktail" upon biliary excretion.

v) Additional studies included the rapidity of rewarming, the portal resistance, the total flow, plasma potassium, aspartate transaminase and plasma haemoglobin. The biliary flow rate under the influence of choleretics was noted in conjunction with the biliary electrolytes and osmolality.

(ii) **Results:**

The rate of transhepatic decrease in platelets is shown in Figure 26. Homologous perfusion of the pig liver with pig blood resulted in a slow transhepatic decrease in platelets which never exceeded 5% of the original. In perfusions of the pig liver with human blood, the transhepatic decrease in platelets was greatest (26%) between 5 and 15 minutes, and resulted in a final circulating count of 30% of the original value. This difference was highly significant.

The other factors measured are shown in Table VIII. The portal resistance was slightly less in homologous perfusions and the total flow rate was slightly greater. The biliary flow rates were similar in both groups of perfusion and were markedly increased in comparison with previous work. Under the influence of added insulin, the plasma potassium was noted to fall slightly in heterologous perfusion, and considerably in homologous perfusion. Although the aspartate transaminase increased similarly in both groups, a greater rise was noted in plasma haemoglobin in heterologous perfusions.

(iii) **Interpretation:**

These results show clearly that while the circuit and oxygenator may have played some role in the clinical thrombocytopenia, the most marked response seemed to result from perfusion of the pig liver with human blood in the same time interval, little biochemical difference was observed. The results seemed to emphasise the role of the liver in the phenomenon of thrombocytopenia, and hence a search was made of the relevant literature to

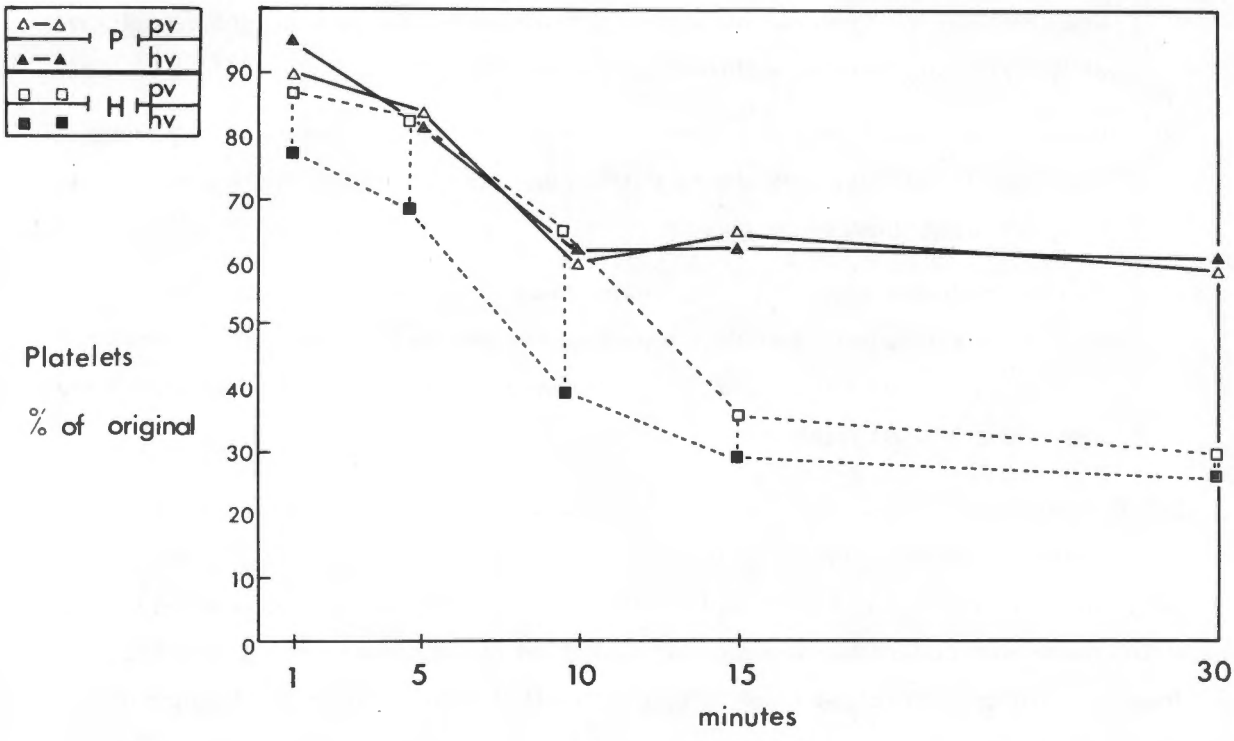


Figure 26 : The mean trans-hepatic levels of platelets in perfusions with porcine or human blood.

P indicates the use of porcine blood

H indicates the use of human blood.

p indicates portal blood

h indicates hepatic venous blood.

		Pig 0 - 1 hr	Human 0 - 1 hr
1) Aspartate transaminase units		+27 (10-50 / 35-90)	+16 (5-25 / 20-35)
2) Plasma osmolality mosm/1		+21 (268-302 / 284-321)	+24 (195-330 / 273-387)
3) Plasma haemoglobin mq/100 ml		+15 (13-16 / 20-45)	+28 (5-50 / 21-184)
4) Plasma potassium mEq/1		−2,5 (3,7-6,5 / 2,4-6,7)	−0,4 (6,5-7,2 / 3,5-5,8)
5) Biliary sodium mEq/1		+14 (138-168 / 122-158)	± 8 (156-164 / 146-150)
6) Biliary potassium mEq/1		−0,9 (3,7-9,0 / 3,8-9,8)	+1,2 (5,2-5,7 / 5,2-3,9)
7) Bile osmolality mosm		+25 (294-300 / 315-330)	+ 7 (285-377 / 279-398)
Bile flow rate ml		7.0	9.0
Bile flow rate ml		7.0	9.0
Total flow ml/kg/min	0	0.28	0.39
	¼	0,30	0,43
	½	0,50	0,55
	¾	0,78	0,63
	1	0,78	0,68
Portal Resistance units	0	0,109	0,091
	¼	0,098	0,071
	½	0,038	0,063
	¾	0,033	0,053
	1	0,028	0,050

Table VIII : A comparison of mean values of the factors studied during one hour perfusion of the pig liver with pig blood (3 experiments) and human blood (5 experiments). In determinations 1 - 7, the mean difference over 1 hour is shown — + indicates a rise and - indicates a fall.

determine the possible nature of the response.

B. THE NATURE OF THE RESPONSE:

Several factors were thought to be inter-acting in provoking this response.

1) The pig liver perfused with human blood constituted a heterograft preparation albeit only for a short period.

2) The circuit itself contributed some degree of platelet destruction although this was a minor component of the response.

3) The liver, apart from being the antigen in the immunological response, has two other relevant roles - one in the process of coagulation and the other in the large phagocytic area of the reticulo-endothelial system formed by the Kupffer cells.

There is a great overlap in all aspects of these factors, but they will be arbitrarily discussed according to the above tenuous division.

1) **The nature of the heterograft response:** (222)

Since the first description of the Forssman antigen, it has been known that "heterophile" antigens occur widely in micro-organisms and animal tissue and their existence may be due partly to accidental correspondence of immunogenic and haptenic determinants in cells and tissues that would otherwise have no close systematic relationship" (63). The antibodies of the human ABO system are thought to result from exposure to a variety of cross-reacting antigens (219). Hence heterophile phenomena have been described in one instance as immunological reactions in which the interaction of antigen and antibody is seemingly non-specific (34).

"The strength and nature of the immune response which may result in damage to or death of the transplant are determined in part by the chemical nature of the surface configurations, the degree of disparity from those of the host, their concentration and distribution on the outer and inner cell membranes and whether or not they are expressed on all cells comprising the graft. The effect of the immune response on the graft is a function of the physicochemical characteristics of the antibody, the ratio of cellular immunity to serum antibody and the degree of heterogeneity of the combining sites of antibody or immunocompetent cell. The nature and intensity of the host response will also be influenced by any prior immunisation of the host to antigens present in the graft" (59).

Attempts to clarify the complexity of the transplant phenomenon between animals of different species have included the use of the terminology "closely related or widely divergent" (208), or more recently by Calne (39) "concordant or discordant" species.

There has been some debate as to whether the response to antigen in the presence of pre-

formed antibody constitutes a reaction of the Arthus or the Schwartzmann phenomenon type, or a combination of both. In kidneys, it has been postulated to be the Schwartzmann phenomenon (140) and in the generalised Schwartzmann phenomenon, there is evidence that platelets are involved (201). But, from the general description of the Arthus reaction, this would appear more apposite to the observations made. In this reaction (45), following deposition of antigen/antibody precipitates on vessel walls, complement is bound and neutrophils and platelets accumulate. Severe injury occurs to the vessel wall, with disruption of the basement membrane, oedema and haemorrhage. Invading neutrophils are said to engulf and degrade antigen/antibody complexes. Mechanisms involved in the localisation of immune complexes in blood vessels include - (i) phagocytosis by endothelial cells especially if the reticulo-endothelial system is already saturated by phagocytosed complexes; (ii) attachment by affinity for vessel wall structures especially if electrostatic conditions are favourable, and (iii) entrapment in the vessel wall as a result of increased permeability induced by these complexes, and their results (46) (see Figure 27).

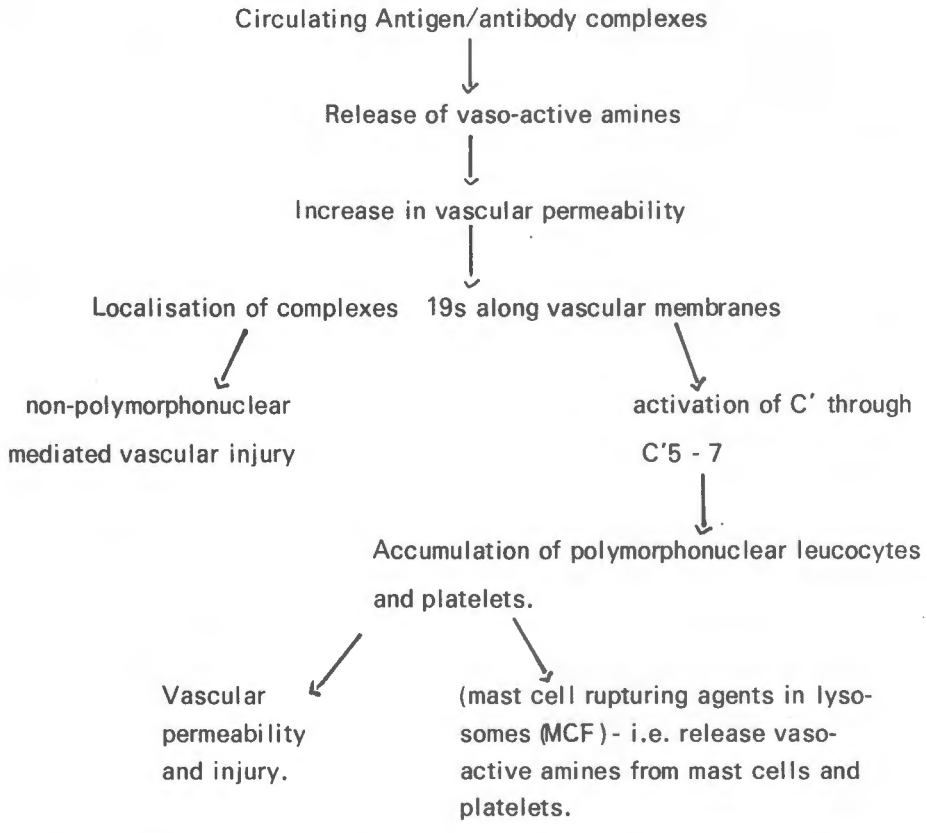


Figure 27 : A hypothetical scheme to represent the effects of circulating antigen-antibody complexes.

C' indicates the fractions of complement.

Once complexes localise, complement is rapidly fixed, polymorphonuclear leucocytes and platelets are attracted and damage ensues. In immune lysis by complement, small holes are formed in cell membranes which allow ions but not large molecules to diffuse through. This results in equilibration of ionic contents across the cell membrane (201). Since some platelet granules are lysosomes, these also result in tissue injury when platelets become degranulated.

The results of experimental heterotransplants have been described frequently. It has been apparent that the liver plays a major role in the reaction to anaphylaxis resultant upon the challenge of a pre-sensitised animal. As early as 1923, in pre-sensitised dogs, the injection of antigen resulted in increased perfusion resistance and decreased flow in the portal system (165). In addition, the effect of anaphylaxis upon platelet counts in the heterograft phenomenon, has been known since 1941 (143).

In this laboratory, (259) the baboon liver and kidney were transplanted into individual recipient pigs. The observation was made that while the kidney was obviously rejected within 30 minutes, becoming turgid, and black with gross proteinuria, the liver continued to perfuse for a much longer period of time. Recipients of these livers recovered consciousness and were alert for up to 18 hours; thereafter they succumbed with a metabolic acidosis which could not be reversed. This syndrome was reminiscent of the totally hepatectomised animal which also survived for a similar period of time, and in which the terminal syndrome also could not be reversed. Histological evidence of damage to the heterografted liver occurred within the first 30 minutes, with sinusoidal congestion and the presence of polymorphs within the sinusoids. Fine non-lipid vacuolation of cells was also seen, with hyaline intracytoplasmic inclusions. There was some nuclear pyknosis and cytoplasmic shrinkage characteristic of parenchymal cell damage.

The association of decreased platelet and polymorphonuclear leucocyte levels in the heterograft process has been frequently described in heterotransplants and heterogeneic perfusions of the kidney (48,168,210) and follows the pattern as described for the localisation of immune complexes (Figure 27). Little work has been performed on the platelet responses to liver heterografts but the few reports record a similar pattern of the sharp decline in platelets within a few minutes of commencement of perfusion. The decline was associated with a fall in leucocytes, complement and cytotoxic antibody (27).

2) **Platelet aggregation:**

Platelet function and the phenomenon of platelet aggregation have been exhaustively reviewed (167,170) in attempts to elucidate the problems of normal and abnormal blood coagulation. For the purpose of this study only the selected references of direct importance will be cited.

Platelet functions have been classified as the support of endothelium; aggregation and the promotion of coagulation; and the activation of prothrombin complex to thrombin (282). Also, the mechanism of aggregation has been widely studied. Aggregation appears to be dependent upon ADP released from erythrocytes (86) which is dependent upon the availability of glucose (139). It is thought that the reduction of ATP to ADP on the surface of platelets and red cells results in a negative charge which increases the tendency to adhere (30). Aggregation may be induced by ADP, adrenaline, 5-hydroxytryptamine, thrombin, collagen and fatty acids in vitro (171), and the ultrastructure of the changes involved has been recently described (251).

Of greatest significance to this study are the reports of platelet adherence to collagen (158,201), to vascular endothelium (180), and the involvement of platelets in the formation of immune complexes (206). This will be cited in more detail in the next section. Upon aggregation of platelets, various factors are released - histamine, serotonin and lysosomal enzymes, which aggravate endothelial and cellular damage and establish a vicious cycle (131). Local release of adrenaline activates the mechanism by further release of ADP (197).

3) **The role of the liver**

a) The reticulo-endothelial system - It has recently been observed that homologous platelet aggregates could be cleared from the circulation by attachment to an isolated perfused liver, thereby preventing pulmonary damage (195). Clearance of fibrin aggregates has also been noted (89). This highlights the "scavenging" and protecting effect of the R-E system in the liver - the major physiological host defence system (230). In the problem of removal of platelets and immune complexes, the fact that the liver has this large area of macrophages may be significant. While the Kupffer cells have been noted to enhance the action of weak antigens, their role in the immune response is less clearly defined (290). However, in a review of the functions of the R-E system and studies of suppression of phagocytosis by colloidal carbon (276) the question arises whether the role of platelets is indeed to surround foreign particles and transport them to the R-E system hence being phagocytosed themselves.

Overall, the role of the R-E system in regard to the Kupffer cells has been suggested to be (i) phagocytosis of colloidal particles, red cells, viruses, bacteria, lipids and cholesterol, and (ii) the removal of immune complexes and the clearance of antigen. In addition to this role of the Kupffer cells, endothelial cells of the portal and hepatic veins and the hepatocytes are also capable of these clearance functions (21).

b) Endothelial surfaces and tissue factors - Damage to the vascular endothelium specifically has been of interest to those investigating the nature of thrombogenesis, but in the case of perfusion or transplantation (127) of the liver, where damage to vascular linings may result from inefficient preservation, ischaemia or the mechanical trauma of perfusion or flushing, it

may play an important role in the development of thrombocytopenia. The phenomenon has been described as platelet cohesion to each other, but adherence to connective tissue (249), and different physicochemical properties are involved. Contact with sub-endothelial tissues has been thought to lead to the activation of prothrombin (17). In recent studies it has been shown that it is contact only with collagen and connective tissue from organs with high oxidative phosphorylation ratios (e.g. liver, brain, kidney and muscle) which leads to aggregation of platelets. Contact with tissue homogenates of a number of other organs did not have this effect (265). The resultant aggregation is probably still mediated through ADP, the tissue specific inducer (266); calcium is also required. Repeated leakage of ADP and collagen from disrupted, ischaemic or intoxicated tissues could result in persistence of platelet thrombi, stasis and the development of a vicious cycle (266).

As mentioned previously, it has been suggested that the platelets have a role in "nurturing" the endothelium (94). This has been demonstrated in perfusions of rat thyroid with platelet rich media, using apparatus which was entirely silicone rubber and therefore non-traumatic. In addition, it has been suggested that platelets enter to block endothelial gaps, but that as repair takes place, this attachment of platelets is reversed (83).

c) Coagulation - The liver is reported to play a role in fibrinolysis which may become defective with inadequate preservation as evidenced upon transplantation. Its role has been postulated to be to compensate particularly for the increased fibrinolytic activity after trauma (11). This is confirmed by studies after total hepatectomy in dogs in which an increase in proteolytic activity was noted in the blood with breakdown of fibrinogen to fibrin degredation products with consequent disseminated intravascular coagulation (258).

As mentioned previously, the liver has been supposed to synthesise many of the coagulation factors (283) and in isolation has been shown to remove fibrin deposits from the circulation in its "scavenging" role. In addition, the liver is thought to be the primary site of synthesis of the C'3 fraction of complement (14). Most of these activities are revealed in patients with liver disease, especially in massive liver necrosis (278).

d) Another role of the pig liver which has been shown by the allo-transpantation experiments of Calne (40), is the suggested immunological effect by which it may *protect* other transplanted organs from rejection.

PHASE VI:

Consequent upon this review, a study was designed to investigate the association of other features of the heterograft phenomenon with the already observed thrombocytopenia.

(i) **Design:**

i) Four experiments were performed with human blood circulating through the circuit alone.

ii) Four experiments were performed with the pig liver perfused with pig blood, and

iii) Seven experiments were performed with the pig liver perfused with human blood of various groups - A (2); B (2); AB (3).

iv) The liver was "massaged" by movement of the diaphragm which was mobilised by the motor shown in Figure 5. This procedure was commenced in an attempt to reduce stasis on the dependent surface of the liver which had been noted during previous perfusions.

v) Since these were the first experiments with human blood using the Travenol oxygenator for four hours, a full biochemical evaluation was performed as described in experiments in phase II.

vi) Platelets were counted in samples taken from the portal inflow and the venous return. The results were corrected for the small changes in haematocrit, and were expressed as a percentage of the original pre-perfusion count. The difference between in and outflow platelet counts was designated the transhepatic difference.

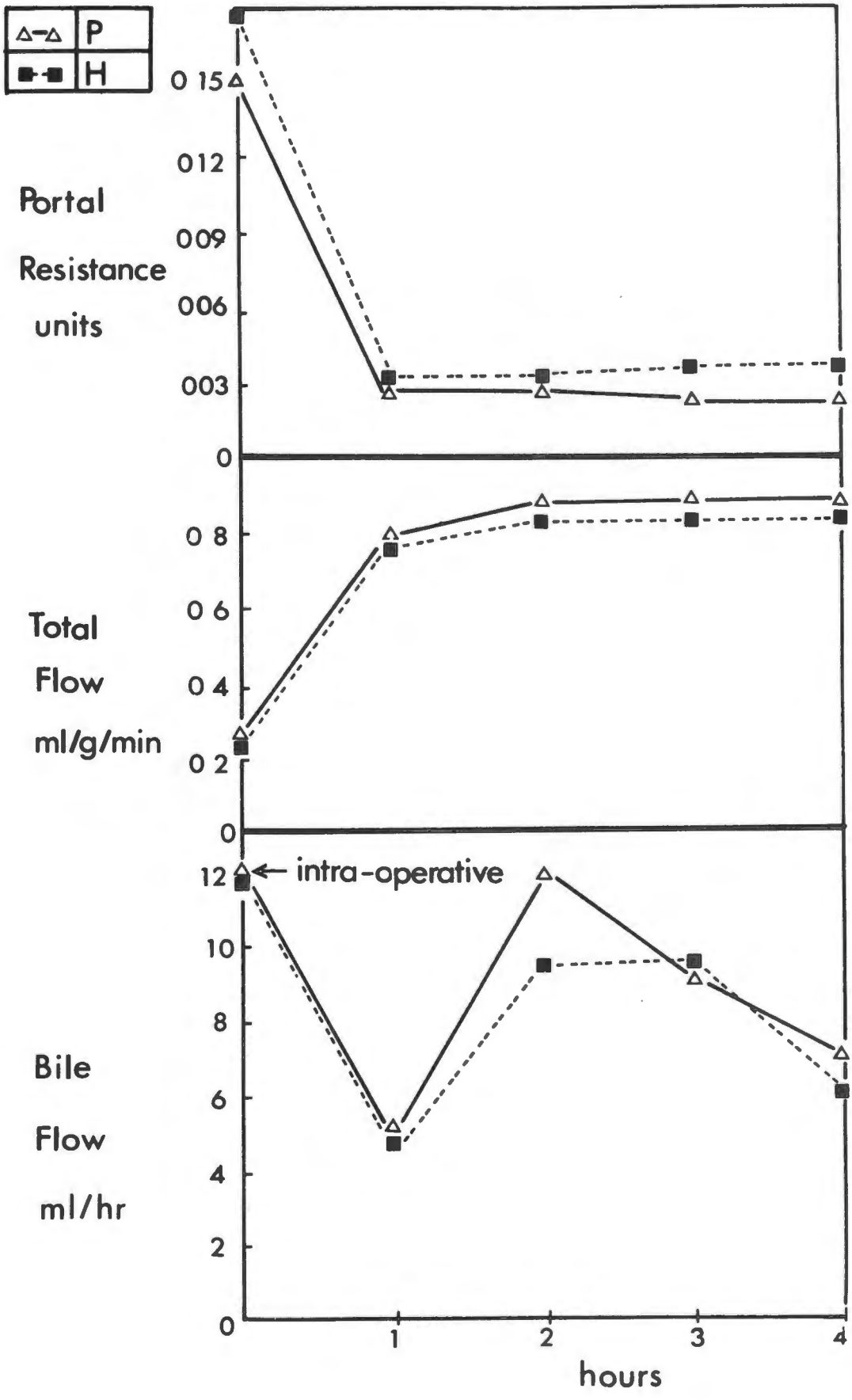
vii) Lymphocytes and leucocytes for cytotoxic tests were generally obtained from the donor of the liver, but in a few instances, multiple results were obtained with the cells of a single donor. Lymphocytotoxic antibodies were sought using the microtechnique of Van Rood (272) and leucocyte aggregating antibodies by the techniques of Kissmeyer Nielsen and Kjersbye (141). Heterophile antibodies were measured in pig plasma against human, baboon, rat, sheep and guinea-pig erythrocytes, and in human plasma against pig, rat, sheep and guinea pig erythrocytes by standard techniques (see Appendix of Methods reference 18).

(ii) **Results:**

a) Physical and biochemical

In Figure 28 the portal resistance, total flow rates and rate of bile flow in perfusions of the pig liver with pig blood or human blood are shown. There was no marked difference between the two groups in the resistances, although the readings in the human group remained a little higher than those in the pig group. Bile flow rate was similar in both groups and was higher than in previous perfusions due to the addition of choleretics, but in 3 of the 7 perfusions with human blood, bilirubin accumulated in the plasma after one hour of perfusion.

Figure 28 : The portal resistance, total flow rates and rate of bile flow in perfusions with porcine or human blood.


P
H
0 15
012
009
006
003
0
Portal
Resistance
units
0 8
0 6
0 4
0 2
0
Total
Flow
ml/g/min
12
10
8
6
4
2
0
intra-operative
Bile
Flow
ml/hr
1
2
3
4
hours

In Figure 29 the levels of plasma and biliary potassium are compared, and although the levels in pig blood were higher, the patterns appear similar. In Figure 30 the highly significant rise of aspartate transaminase in perfusions with human blood may be seen ($p<0,001$) the clearance of the lactate administered at three hours appears similar in both groups although the initial lactate/pyruvate ratios were higher in perfusions with human blood. The rates of clearance of ammonia and increase in plasma urea are shown in Figure 31 from which it may be seen that there is a greater increase in urea levels in perfusions with pig blood, and a more rapid clearance, at least within half an hour, of the administered load.

The appearance of the liver is impossible to quantitate, but it is noted that livers perfused with human blood tended to be blotchy and to develop small black areas of ischaemia after four hours' perfusion which were not seen in the livers perfused with pig blood.

b) Haematological and Immunological

From Figure 32 it may be seen that there was no significant decrease in platelets during perfusion of human blood through the circuit alone. During homologous perfusion of the pig liver with pig blood, the maximum mean transhepatic decrease (6-8%) occurred at 1 and 5 minutes, and between 15 and 30 minutes there was a reversal of the levels. This was presumed to be due to release of platelets after initial adherence within the liver.

During heterologous perfusion with human blood, the mean transhepatic decrease in platelets increased from 24% at one minute to a peak of 57% at 5 minutes. This resulted in a final total circulating level of 20% of the original value at 30 minutes, which was highly significantly different from the level of 60% obtained in homologous perfusions. No subsequent increase of circulating platelets was noted in the remaining 3½ hours of perfusion. A similar observation was made in the leucocyte counts. In homologous perfusions, a mean transhepatic decrease of 9% and 3% was noted at one and five minutes, and an increase of 20% and 3% at 15 and 30 minutes. In heterologous perfusions, the mean trans-hepatic decrease increased from 15% at one minute to 39% at 5 minutes with a final total circulating level of 15%.

The levels of complement fell to 94% of the initial value in perfusions of human blood alone; to 95% in perfusions of the pig liver with pig blood, and significantly to 72% in perfusions of the pig liver with human blood ($p<0,001$). The levels of plasma haemoglobin increased in all perfusions, with a greater increase in human blood perfusing pig livers.

Lymphocytotoxic, leucoagglutinating and heterophile antibodies in human blood diminished within five minutes and were almost totally absent after 30 minutes of perfusion through the pig liver. No alteration of these antibodies occurred in the human blood when perfused through the circuit alone, nor in pig blood perfused through the pig liver.

c) Histology

Histological features were similar to those reported in the comparison of livers perfused with porcine or human blood in phase II of these experiments. Whilst the architecture seemed reasonably well preserved after four hours' perfusion with porcine blood, with only occasional evidence of sinusoidal congestion and haemorrhage, these features were marked in livers perfused with human blood. In two sections, frank total cell necrosis was seen in areas.

(iii) **Interpretation:**

One observation made in these experiments was that there appeared to have been a reduction in the levels of portal resistance in perfusions with human blood as compared with experiments in phase II. No reasons could be advanced for this observation except that the total flow in these experiments was also greater than in phase II; this observation may only reflect the lesser resistance rather than explain it. The differences in the experimental design of this group and phase II lay in the oxygenators, and in the infusion of the "cocktail". It could be postulated that less damage occurred to the human blood when passed through the Travenol oxygenator, with fewer small particles to lodge in the portal vasculature, or that the infusion acted as a vaso-dilator. Bile acids are known to have vasodilatory properties, but it is likely that there would have been some obvious effect upon the homologous resistance in addition to that in heterologous experiments. These results were the mean of 7 perfusions with different blood groups - of these, only 3 were of group AB, which had been previously noted to cause the greatest rise in portal resistance and aspartate transaminase. Hence the lower resistances may be evidence of random use of blood groups and the result of taking mean values.

The levels of potassium in perfusions with human blood were reduced over the course of the experiments as compared with observations in phase II. This difference might also be attributed to the use of insulin, and could perhaps indicate that the release of potassium which occurred in the first perfusions with human blood was reversible by infusion of insulin.

Nonetheless there was still evidence of greater liver damage in organs perfused with human blood, there being a highly significant increase in the levels of aspartate transaminase. It should be noted also that there was a lesser increase in perfusions with pig blood in these experiments than had been observed in perfusions in phase II. This might be attributed to the improved perfusion of the liver as a result of the diaphragmatic movement, which prevented obvious stasis.

As in phase II, there was clearance of the administered load of lactate and ammonia within one hour of infusion. Livers perfused with pig blood appeared to synthesise more urea during the first three hours of perfusion, but in response to the load, the levels of plasma urea increased equally in both groups within the hour tested.

Figure 29 : The levels of plasma and biliary potassium in perfusions with porcine or human blood.

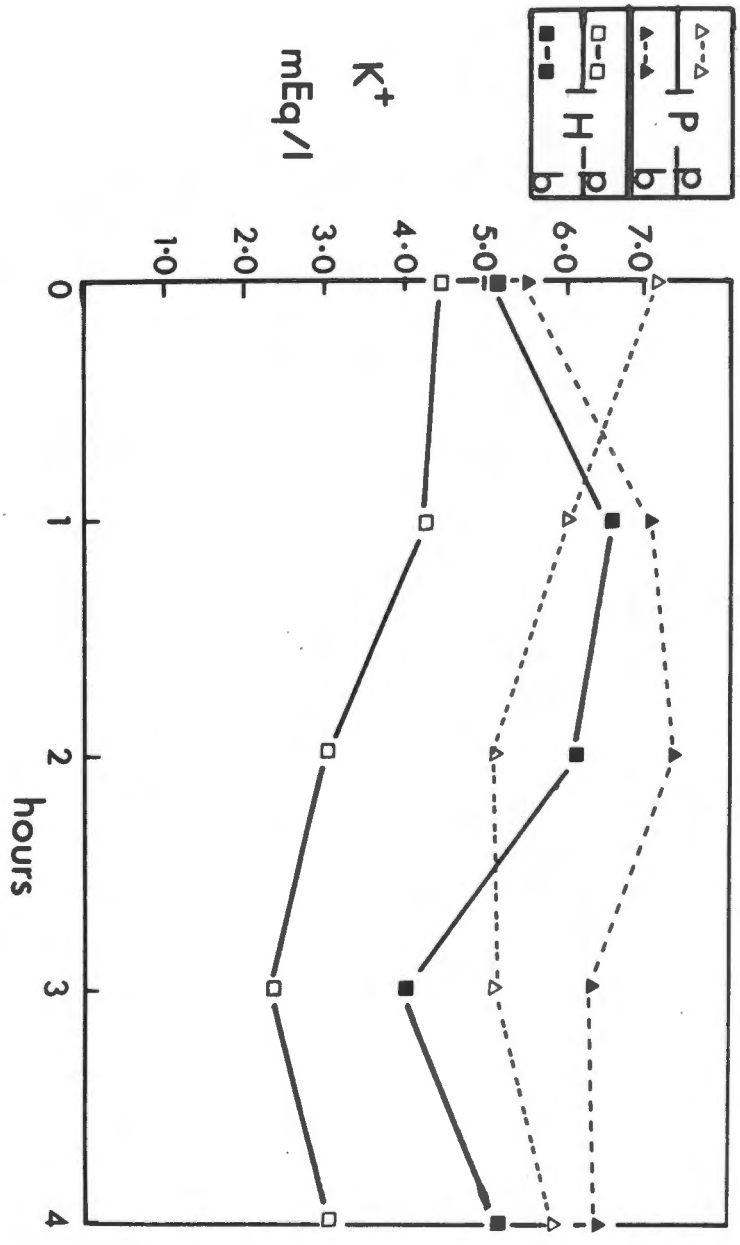
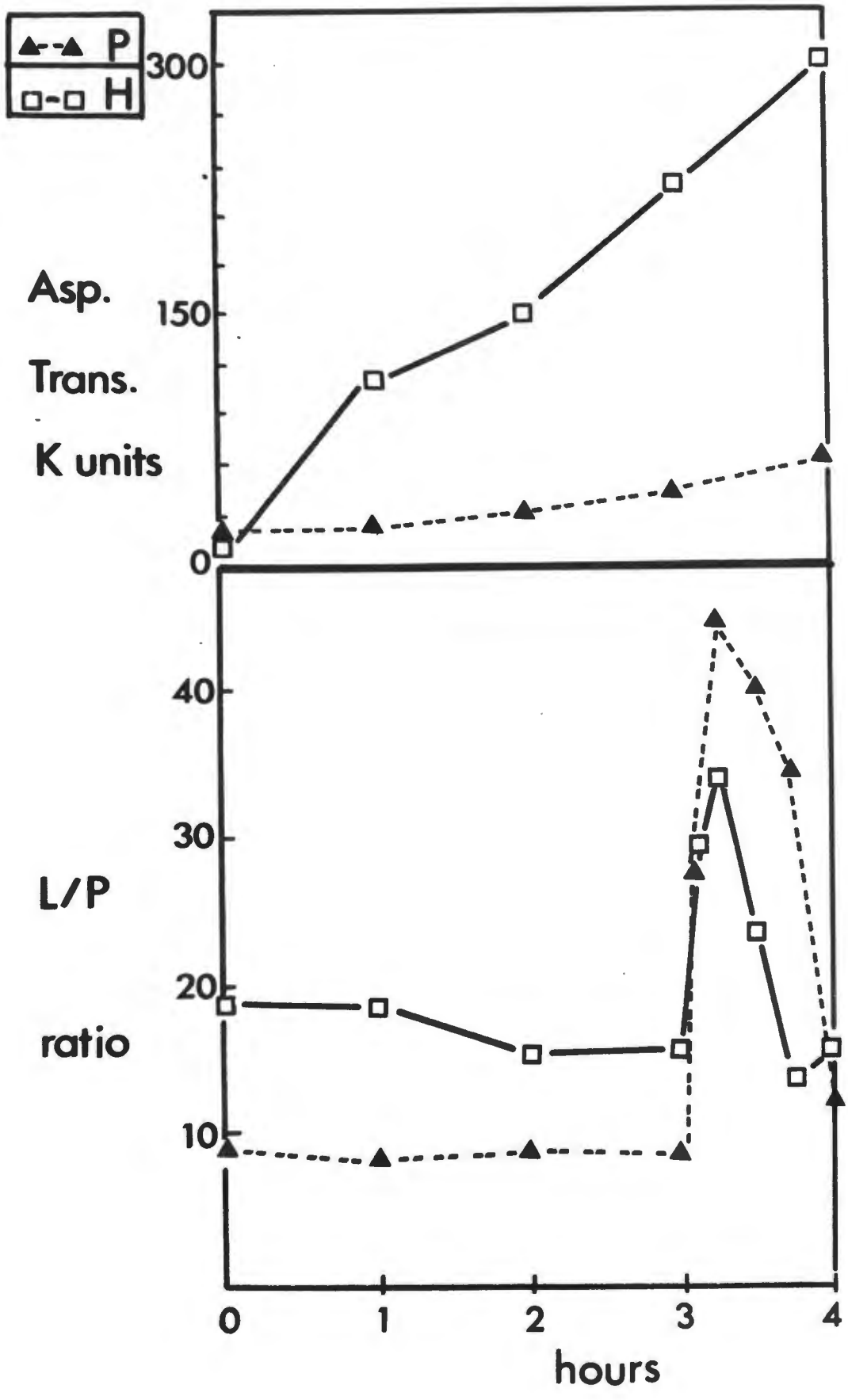

P p
b
H p
b
K+
mEq/l
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
1
2
3
4
hours

Figure 30 : The levels of aspartate transaminase and the clearance of lactate in perfusions with porcine or human blood.


P
H
300
150
0
Asp.
Trans.
K units
40
30
20
10
L/P
ratio
0
1
2
3
4
hours

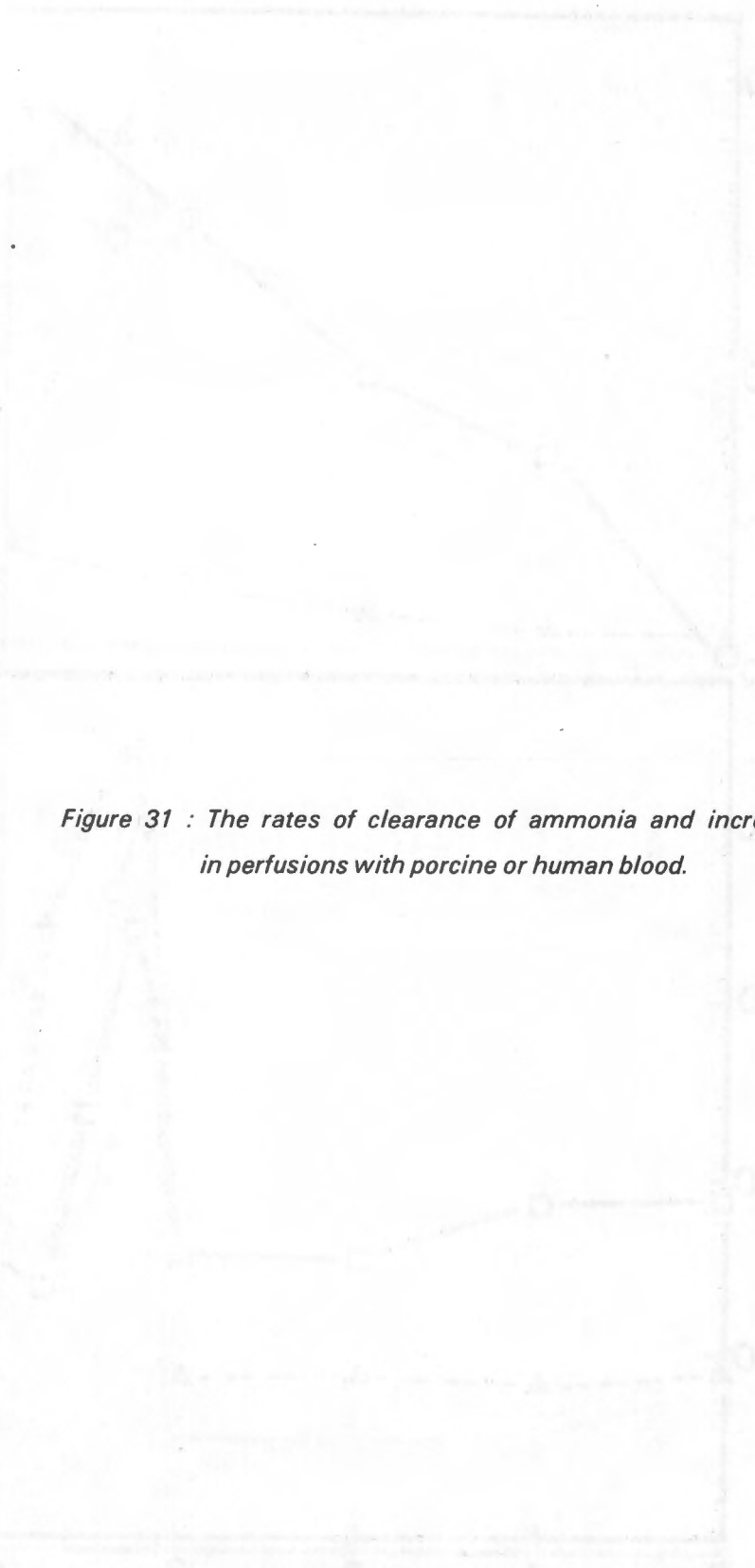
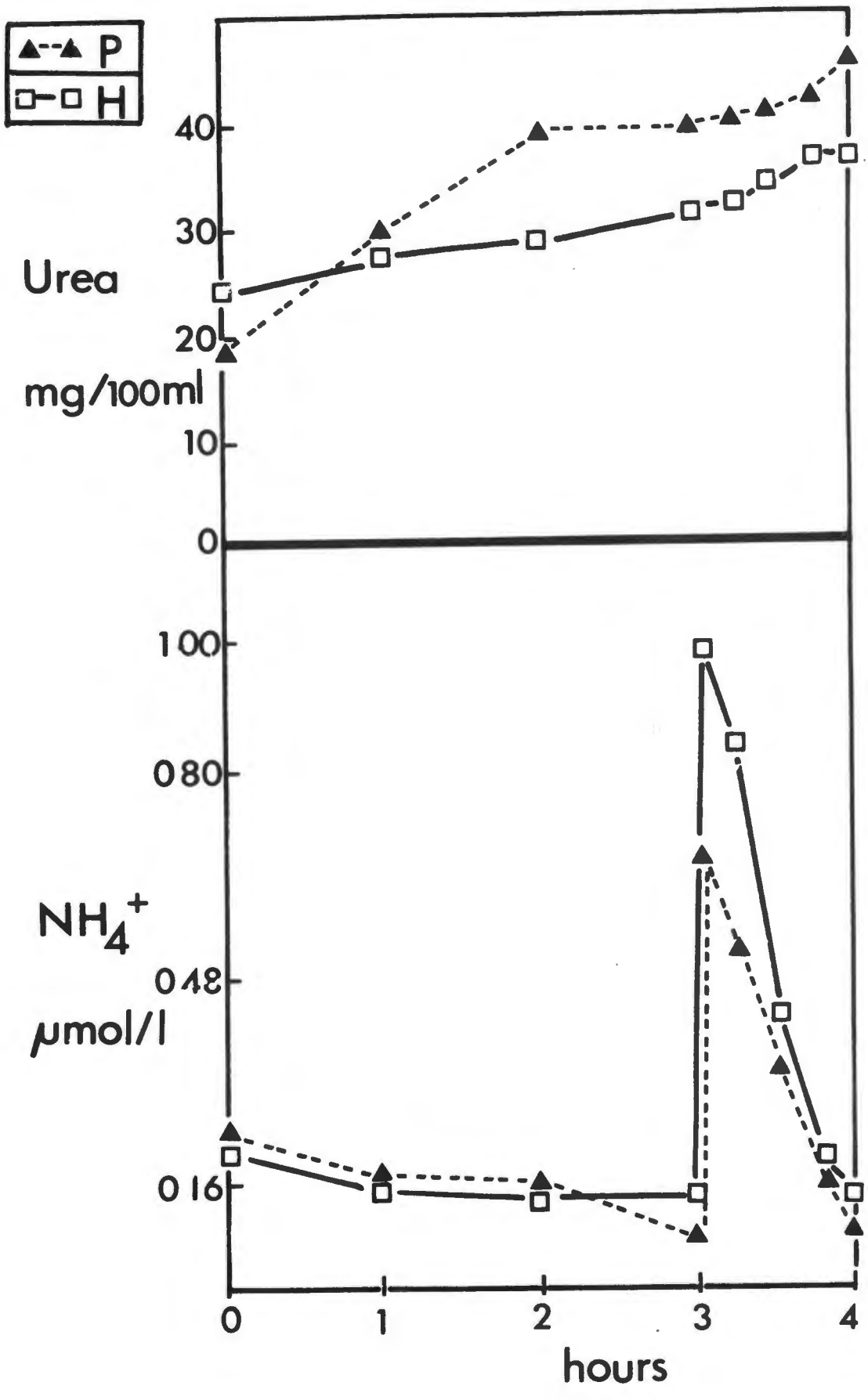


Figure 31 : The rates of clearance of ammonia and increase in urea in perfusions with porcine or human blood.


P
H
40
30
Urea
20
mg/100ml
10
0
100
080
NH_4^+
048
µmol/l
016
0
1
2
3
4
hours

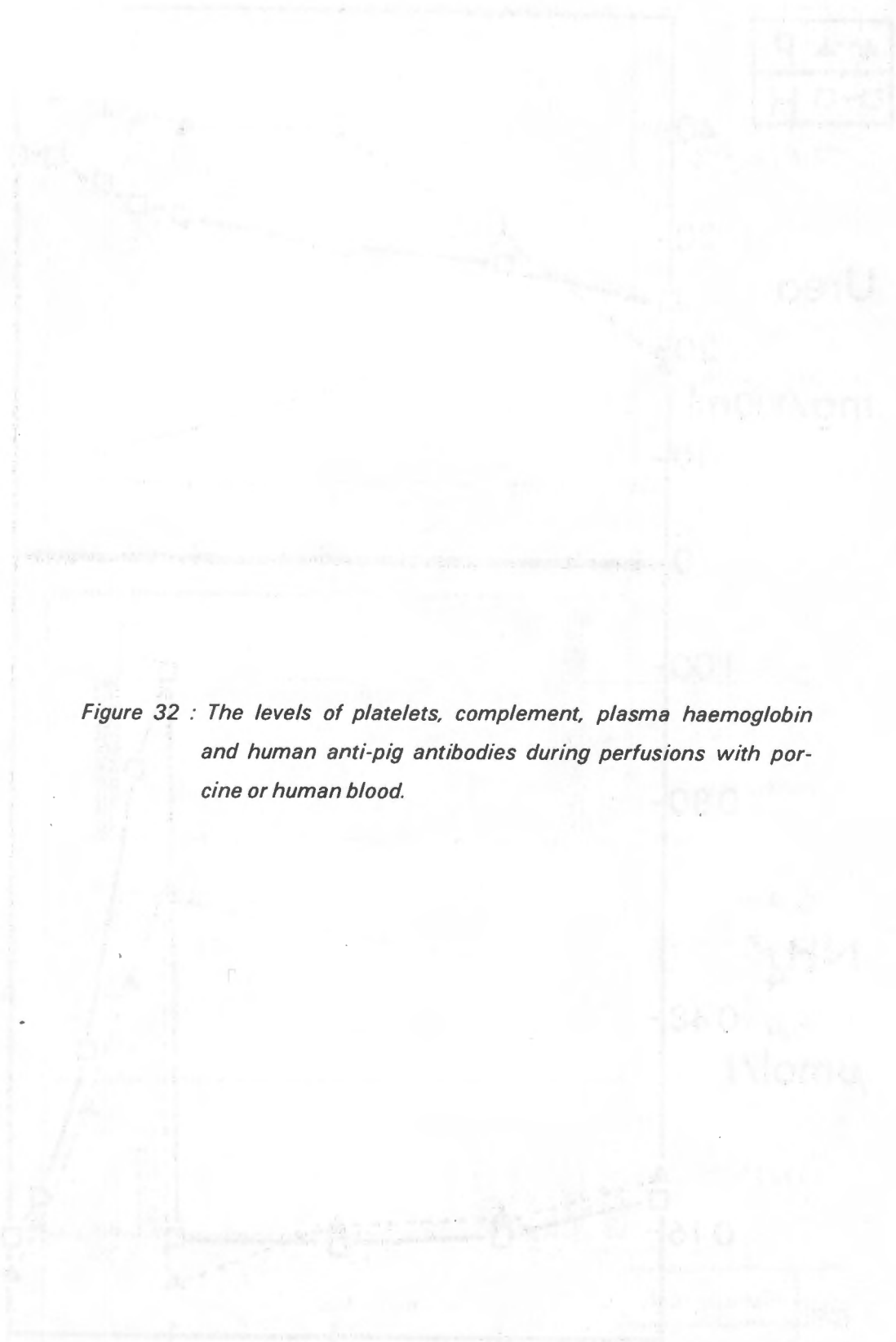
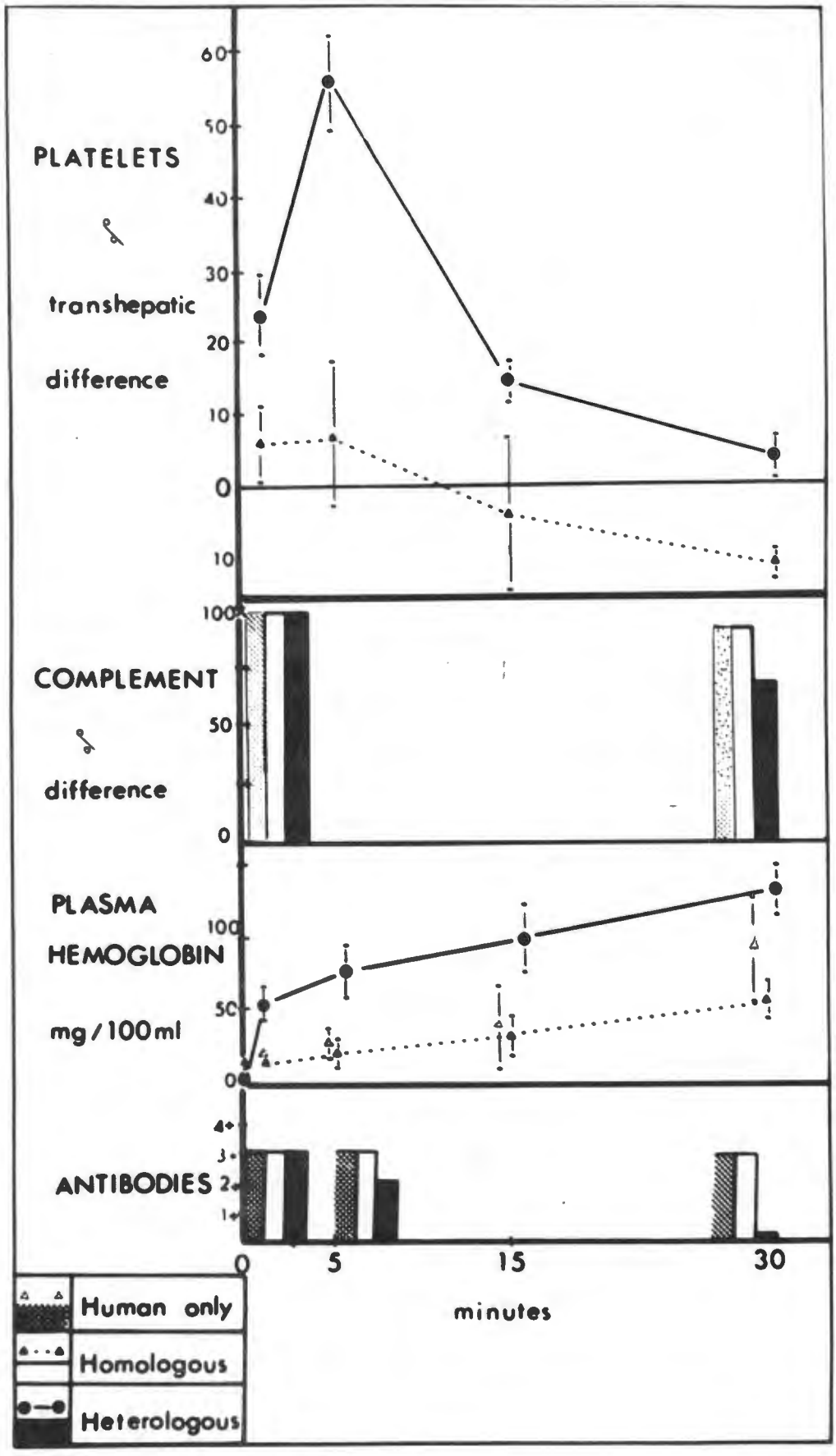


Figure 32 : The levels of platelets, complement, plasma haemoglobin and human anti-pig antibodies during perfusions with porcine or human blood.


PLATELETS
transhepatic
difference
60
50
40
30
20
10
0
10
COMPLEMENT
difference
100
50
0
PLASMA
HEMOGLOBIN
mg/100ml
100
50
0
ANTIBODIES
4+
3+
2+
1+
0
5
15
30
minutes
Human only
Homologous
Heterologous

Hence there was evidence of liver damage in the perfusions with human blood in both phases II and VI. In phase VI a reversal of potassium leakage occurred probably as a result of the addition of insulin, and a change in the pattern of the portal resistance which was attributed to the infused "cocktail" or to the various blood groups used. The overall appearance and perfusion of the liver was markedly improved by the use of the diaphragm.

The patterns of platelet and leucocyte decrease across the liver and the decline in the levels of complement and antibodies confirmed the proposed heterograft nature of this response.

Subsequent investigations were made in an attempt to prevent this response.

CHAPTER SEVEN

ATTEMPTS TO PREVENT THROMBOCYTOPENIA

Introduction (1) Prevention through the mechanism of the aggregating process — drugs
— calcium

(2) Prevention with colloidal or particulate matter

(3) Attempted prevention in the heterograft model

Experiments Introduction

Phase VII

(i) Design - (A) Homologous perfusion - attempts to improve endothelial damage.

(a) Temperature
(b) Changes in solutions
(c) Drugs
(d) Non-starvation

(B) Heterologous perfusion - attempts to prevent platelet aggregation on the basis of trauma ADP release or the immunological phenomenon.

(a) Hydrocortisone
(b) Trasylol
(c) Periactin
(d) Cold
(e) Heparin
(f) Persantin
(g) Colloidal carbon
(h) KRB + KG
(i) (h) + heparin
(j) (h) + EDTA
(k) KRB + KG + McCoys 5A
(l) Human blood, and plasma flushes
(m) 2 pig livers successively

Justification of design

(ii) Results -(A) Homologous

(a) Effect of increasing temperature of flushing solution

(b) Effect of changing constitution of flushing solution

(c) Effect of added drugs

Interpretation

-(B) Heterologous

Interpretation

Introduction

A wide variety of methods has been employed in an attempt to prevent platelet aggregation in vitro and in vivo. Many of these have been successful in vitro, but have failed in the in vivo heterograft.

(1) **Prevention through the mechanism of the aggregating process**

Two principal features in platelet aggregation are the release of ADP, and the requirement for calcium. Many attempts at prevention of aggregation have been directed at these two factors. (Few reports exist of each of these methods and hence a number of references will be cited. An attempt has been made however to select methods with a possible clinical application).

Drugs which have been used to inhibit aggregation include anti-ADP enzymes, local anaesthetics, antihistamines, tranquillizers and anti-depressant drugs (171). Adenosine monophosphate has been used in its role of replacement of ADP (30). Since the energy of oxidative phosphorylation is necessary for ADP-induced aggregation, the effect of removal of glucose has been explored. Antimycin, oligomycin and 2-4 dinitrophenol have been used (139) for prevention of the synthesis of enzymes involved in oxidative phosphorylation. Acetyl salicylic acid is thought to be effective by causing the incorporation of acetate into the metabolic pathway (13). The pyrimido-pyrimidine compound RA-233 has been used to inhibit calcium and ADP-induced aggregation and to decrease the release of platelet factor 3 by kaolin (109), whilst the related drug, Dipyridamole (Persantin) has been found to slow the disappearance of adenosine (75). Arginine methyl esters have also been used empirically to prevent platelet aggregation (182). Sulfinpyrazone and butazolidine have caused an alteration of the response of platelets to surface stimulation, by blocking the aggregating action of collagen, antigen/antibody complexes and γ-globulin coated surfaces on platelets. This is also one of the multiple functions of prostaglandin E_1 in vitro (138).

Calcium plays a vital role in clotting, and aggregation of platelets has been suggested to be independent of any other clotting factors except perhaps thrombin (15). The release of histamine from rabbit platelets has been used as an indicator of platelet aggregation or its prevention. This reaction has been found to be dependent upon specific levels of calcium and the pH. The range of pH has been found in vitro to be narrow - between 6,2 and 7,8, and absolute removal of calcium by chelation with EDTA or citrate has resulted in inhibition of platelet aggregation (172).

(2) **Prevention with colloidal or particulate matter**

Infusion of long-chain fatty acids has been shown to lead to thrombosis (52), but unsaturated fatty acids were noted to be less active (294) and mixture with serum before injection also

seemed to decrease the effect. Other lipids - e.g. ethyl esters of oleic and stearic acid seem capable of blocking aggregation (21,255). Colloidal carbon has been used to block the reticulo-endothelial system but with incomplete blockade, compensation occurs by the remaining unblocked areas of the macrophages (21). However, it has also been suggested that since platelets are required for the transport of colloidal particles to the reticulo-endothelial system (276), the apparent blockade occurs as a result of thrombocyopenia or inefficient action of platelets.

(3) **Attempted prevention in the heterograft model**

Heparin and aspirin have been used for their anticomplementary effect and reversal of platelet aggregation in attempts to prevent rejection of the heterografted kidney (160), and a large number of other drugs has been explored (166). The use of anti-lymphocyte serum, blood grouping and cross matching or pre-treatment with donor antibody has been found ineffective in such experiments (208). Cyproheptadine (Periactin) has been used with success in preventing platelet aggregation and vasculitis in heterografted kidneys (38), but the anti-fibrinolytic and anticomplementary effects of Arvin and heparin, and the use of anti-platelet serum were ineffective. However, in these same experiments, the perfusion of the recipient's blood through an isolated liver resulted in reduction of platelet and cytotoxic antibody levels and prolongation of the heterograft survival (248). In vitro studies with human platelets and heterologous anti-platelet antiserum have shown that the sulfhydryl reducing agents monoiodoacetate and N-ethyl maleimide prevented aggregation of platelets (182).

In a more recently described technique, some success has been reported with mechanical separation of plasma fractions. The attachment of the circulation of animals to a forced flow electrophoresis apparatus resulted in the removal of substantial amounts of antibody and caused prolonged survival of heterologous kidney grafts (27,244).

In the following experimental approach to this problem, two directions were taken.

(i) Attempts were made to reduce the endothelial damage by manipulation of the method of short-term preservation of the liver after removal, and its flushing. The experiments of Dawkins et al (58) as previously reported, were taken as a basis for these studies.

(ii) Several of the means detailed above were selected in an attempt to prevent the platelet response following heterologous exposure.

PHASE VII

(i) **Design**

A - Attempts to reduce endothelial damage in homologous perfusions

a) Variations were made in the *temperature* of the flushing solution - temperatures of 4, 20 and 38°C were used. In the last instance, the liver lost heat during transport to the perfusion laboratory and before commencement of perfusion when the mean temperature was 26°C. Livers which had been perfused with the 4°C solution as before, gained heat to a mean of 10°C. The livers perfused at 20°C tended not to change temperature since this was approximately the ambient temperature.

b) Variations were made in the *constitution of* the flushing solution and the solutions used to prime the circuit and to infuse during perfusion. Following the work of Dawkins (58), a modified Krebs Ringer bicarbonate solution (see Table III) was used to flush the liver and to prime the circuit, and several substrates were added to the flushing solution, the prime and the infusing solution. These substrates included fumarate, pyruvate and glutamate in one experiment, and in several experiments, α-oxoglutarate which had been found the most effective by Dawkins in the preservation of oxidative phosphorylation. The concentration of α-oxoglutarate chosen was 15mM being divided equally between the flushing solution, the prime and the infused fluid, and the substrate was added both to TisUsol and the Krebs Ringer bicarbonate.

A further attempt to improve substrate levels was made with the use of the tissue culture medium McCoys 5A. This had been used for preservation of the isolated spleen (66), and contained "physiological" amounts of numerous amino acids, vitamins and electrolytes.

c) A few of the *drugs* described above were used.

Heparin - in addition to the 45mg/l present in the human blood as obtained from the Transfusion Service, a further 250mg was added initially to the 1,5 litre perfusate, and 250mg was added by continuous infusion over the 4 hours' perfusion. An additional amount of 250mg was added to the flushing solution.

Ethylene-diamine-tetra-acetate (EDTA) was added to deplete the perfusate of calcium, the dose used being 1,0mg/l.

Adenosine (0,001M) was added in an attempt to block the aggregating effect of ADP.

d) The pig donating the liver was *unstarved* in an attempt to ensure adequate pre-operative levels of glycogen in the liver. This lack of starvation made dissection a little more difficult but no obvious additional trauma resulted to the liver.

B - Attempts to prevent platelet aggregation on the basis of trauma, ADP release, or the immunological phenomenon in heterologous perfusions

a) *Hydrocortisone* - 100mg/1,5 litres was added to the perfusate just prior to commencement of perfusion.

b) *Trasylol* - 75 000 units/1,5 litres was used as an antifibrinolytic agent and has also been reported to have effect in the prolongation of heterografts.

c) *Cyproheptadine* (Periactin) was used in the dose 160mg/1,5 litres. The tablets were dissolved in water and the pH corrected to 7,0 with 0,1N hydrochloric acid. The final suspension was filtered to remove the agents used in preparation of the tablet form.

d) The *temperature* of the perfusion was reduced to 10-15°C to investigate whether the platelet aggregation was temperature dependent.

e) *Heparin* was added as in A(c).

f) *Dipyridamole* (Persantin) was used in the dose of 200mg/1,5 litres.

g) A suspension of *colloidal carbon* was prepared in TisUsol in a concentration of 4mg/ml as had been described (131). This was used to flush the liver prior to perfusion.

After evaluation of the results of the experiments in section A, the most efficient combinations were chosen and were used in the heterograft procedure in combination with other pharmacological manoeuvres.

h) The *modified Krebs Ringer bicarbonate solution* was used as a flushing solution and prime, with added α-*oxoglutarate* as described in A(b).

i) As 2h with added *Heparin* as in A(c).

j) As 2h with added *EDTA* as in A(c).

k) Krebs Ringer bicarbonate flushing solution with *McCoys medium 5A* as priming solution and as addition to the infusion solution. α-oxoglutarate as used in A(b) was added.

l) In two different groups of experiments, attempts were made to *block* the *antibody-binding sites* within the liver by prior exposure of the liver to human blood or plasma containing such antibody. Hence, livers were flushed either with fresh human blood compatible with that to be used for priming the circuit, or with reconstituted dried plasma.

m) In a further attempt to *remove antibody* from the human blood, two livers were used, after both had been dissected. The first was inserted into the circuit and allowed to perfuse for 15 minutes while samples were taken, and thereafter, the second liver was inserted into the circuit and the perfusion continued for 4 hours. In this experiment, in retrospect, it was realised that the platelets would disappear with the initial perfusion and might be present in too few numbers to show a significant alteration in the second part of the perfusion.

- Except where otherwise stated, all drug additions were made to the perfusate before commencement.
- In all additions of drugs, the dose chosen was the greatest which might be used in a clinical procedure.
- The first half hour of perfusion was studied in more detail in a number of the above experiments in an attempt to define more clearly the occurrences during the first hour of perfusion in which most of the radical changes take place. The factors reviewed were the portal resistance, the total flow rate, and the rate of increase in the temperature, the plasma potassium and haemoglobin, and the aspartate transaminase.

Justification of Design:

In all experiments, especially those performed with human blood, but also in those using pig blood, only a single experiment of each type was performed unless otherwise stated. If any procedure had appeared to result in a marked difference in the decline of platelets which was taken as the prime objective, further studies would have been made after the safe clinical application had been thoroughly explored. This policy was adopted due to the expense involved in each experiment, and in an attempt to prevent over-use of human blood. No statistical conclusions could be drawn, but provided that experimental conditions were kept constant, a technique was excluded if no improvement resulted in trans-hepatic platelet differences. This applied especially in the pharmacological group in which other workers have reached a similar conclusion in heterograft experiments.

(ii) **Results**

A - Attempts to reduce endothelial damage in *homologous* perfusions

The results obtained in 13 groups of perfusions are shown in Table IX where the platelets and leucocyte counts for the first half hour are recorded. The mean changes in potassium, plasma haemoglobin and aspartate transaminase at thirty minutes and four hours after perfusion appear, and the mean total flow rate, portal resistance and changes in temperature with time are also recorded.

a) Effect of increasing the *temperature* of the flushing solution:

Comparison of the control changes in platelets with those occurring after flushing with TisUsol at 20°C and 38°C shows a marked improvement with the latter two temperatures. The same effect is noted when Krebs Ringer bicarbonate was used at 4°C and 38°C. A greater increase of aspartate transaminase over four hours occurred with either TisUsol or KRB at 4°C, than when the solution was warmer. With KRB at 38°C a lower total flow rate was achieved.

The improvement in perfusion after flushing with warm solution was much more obvious

Table IX : The mean values for temperature change, and for portal resistance and total flow rate during the first 30 minutes of homologous perfusion.

Table IX : The mean values for aspartate transaminase, plasma haemoglobin and plasma potassium at 30 and 240 minutes of perfusion with homologous blood.

Table IX : The mean values of leucocytes in homologous perfusions using methods to prevent endothelial damage.

Table IX : The mean values of platelets in homologous perfusions using methods to prevent endothelial damage.

	(Initial Temp)	Temp. change at			Resistance at:			Total flow at:		
	0''	1''	5''	30''	1''	5''	30''	1''	5''	30''
TisUsol 20°C	(20)	8	13	17	0.10	0.029	0.023	0.27	0.83	1,06
TisUsol 38°C	(30)	0	1	7	0.14	0.050	0.032	0.31	0.60	0.91
KRB 4°C	(24)	7	10	13	0,069	0,037	0,026	0,33	0,55	0,72
KRB 38°C	(27)	2	5	9	0,160	0,190	0,120	0,29	0,32	0,43
TisUsol + α KG	(28)	0	4	8	0,10	0,040	0,026	0,36	0,44	0,56
Fumar/pyruv/glut.	(25)	6	9	12	0,11	0,110	0,060	0,44	0,74	0,99
KRB + EDTA	(16)	12	22	22	0,123	0,091	0,048	0,49	0,38	1,01
KRB + α KG at 38°C	(23)	7	12	14	0,094	0,068	0,052	0,36	0,53	0,65
KRB + α KG + Heparin	(28)	2	6	14	0,107	0,085	0,037	0,38	0,80	0,92
KRB + α KG + EDTA	(23)	7	9	14	0,092	0,091	0,042	0,31	0,60	0,76
Unstarved pig	(18)	8	10	19	0,130	0,174	0,043	0,27	0,26	0,73
KRB + α KG + McCoys	(25)	6	10	12	0,050	0,050	0,040	0,73	0,73	0,96
KRB + α KG + Adenosine	(24)	9	12	13	0,085	0,037	0,048	0,43	0,94	0,94

	Asp.T/sam		Plasma Hb		Plasma K+	
	30''	240''	30''	240''	30''	240''
TisUsol 20°C	5	35	0	20	1.6	3.7
TisUsol 38°C	0	15	2	30	0,8	2,8
KRB 4°C	5	15	0	25	1,6	6,4
KRB 38°C	30	90	40	70	1,0	3,0
TisUsol + α KG	10	25	0	5	0,6	0,6
Fumar/pyruv/glut.	0	10	0	55	0,7	4,2
KRB + EDTA	15	50	3	60	0,5	1,5
KRB + α KG at 38°C ($\bar{6}$)	0	15	3	30	0	2,4
KRB + α KG + Heparin ($\bar{2}$)	0	30	4	12	0	3,8
KRB + α KG + EDTA ($\bar{2}$)	10	50	5	75	0	1,8
Unstarved pig	15	80	10	50	1,0	2,0
KRB + α KG + McCoys	10	30	0	20	1,3	2,1
KRB + α KG + adenosine	5	40	30	60	1,7	1,9
Control	25	-	15	-	0,9	-

	Leucocytes % PV /HV				
	1''	5''	15''	30''	180''
TisUsol 20°C	107/18	74/23	67/32	35/29	-
TisUsol 38°C	71/58	76/12	71/29	29/18	43
KRB 4°C	110/27	75/21	63/26	37/25	19
KRB 38°C	108/15	100/27	38/25	33/22	11
TisUsol + α KG	84/26	87/36	62/43	39/33	24
Fumar/pyruv/glut.	100/55	89/28	85/27	47/33	13
KRB + α KG at 38°C ($\bar{6}$)	84/55	83/33	62/36	48/40	40
KRB + EDTA	87/37	94/19	45/29	25/34	16
KRB + α KG + Heparin ($\bar{2}$)	101/52	101/25	70/25	36/33	25
KRB + α KG + EDTA ($\bar{2}$)	67/40	63/38	50/50	26/42	29
Unstarved pig	86/28	82/23	71/31	45/25	18
KRB + α KG + McCoys	102/20	108/16	68/30	45/36	30
KRB + α KG + adenosine	83/74	72/19	57/40	40/40	19

	Platelets % PV / HV				
	1''	5''	15''	30''	180''
TisUsol 20° C	99/91	110/110	117/114	90/104	-
TisUsol 38° C	65/67	65/53	65/59	61/58	53
KRB 4° C	110/58	80/63	66/56	58/55	50
KRB 38° C	90/54	94/90	83/80	86/81	26
TisUsol + α KG	109/73	108/99	112/98	93/115	67
Fumarate/pyruvate/glutamate	83/67	93/75	98/73	74/70	56
KRB + EDTA	106/58	91/65	51/82	56/98	30
KRB + α KG at 38° C ($\bar{6}$)	78/68	83/71	81/82	70/86	75
KRB + α KG + Heparin ($\bar{2}$)	109/60	87/83	88/86	83/75	62
KRB + α KG + EDTA ($\bar{2}$)	98/68	88/82	93/107	57/107	72
Unstarved pig	65/57	66/56	64/60	47/48	47
KRB + α KG + McCoys	84/76	106/88	84/92	86/96	71
KRB + α KG + Adenosine	91/55	69/93	60/126	93/115	67
Control	90/95	83/82	64/63	59/61	45

in the appearance of the livers. The organs which had been flushed with a warm solution were a homogenous beige colour whereas the livers perfused with cold solution often showed small red areas which looked like bruising. In the first few minutes of perfusion, blood spread diffusely and evenly through the warm-flushed livers whereas there were areas of patchy perfusion for the first hour in livers which had been flushed cold.

b) Effect of changing the *constitution* of the flushing solution/prime:

It is striking that in all experiments in which -oxoglutarate was added, the platelet count after 3 hours was between 62 and 75%, contrasting with the control level of 45%. The levels of leucocytes at 3 hours also tended to be higher, being between 19 and 40%, whereas most of the other readings, except where warm TisUsol was used, were less than 19%. Although no obvious differences were present in any of the other features measured, the improved platelet and leucocyte levels justified the attempted use of α-oxoglutarate in the heterologous perfusion.

c) Effect of added *drugs:*

The use of Heparin in the doses planned seemed to result in additional haemorrhage within the liver during perfusion. No obvious improvement was noted in the three hour platelet or leucocyte counts. Addition of EDTA seemed to result in elevation of the aspartate transaminase and potassium levels but not in improvement of the cell counts. Adenosine was added only in the presence of αKG and no improvement was observed.

Non-starvation of the animal made little difference.

Interpretation:

The conclusions drawn from these experiments were that the use of a warm flushing solution containing αKG appeared to result in improvement of the platelet and leucocyte counts, and in the appearance of more even perfusion.

The use of McCoys medium 5A was found to have a major advantage in regard to the phenol red which is used as an indicator. This substance was readily excreted in bile and its clearance from plasma and appearance in bile was obvious. In the early minutes of perfusion, this may provide evidence that bile is being secreted rather than being a mere transudate of oedema fluid across the biliary membranes. In these homologous perfusions, there was an interval of 3-5 minutes between obvious reduction in plasma levels of dye and the appearance of colour in the bile. This observation should be followed by quantitative colorimetric determination, with the performance of formal clearance studies.

Movement of the diaphragm resulted in improved perfusion of the dependent surface of the liver with absence of the black areas of stasis which had appeared routinely when the diaphragm was immobile.

Table X : The mean values for temperature change, and for portal resistance and total flow rate during the first 30 minutes of heterologous perfusion.

Table X : The mean values for aspartate transaminase, plasma haemoglobin and plasma potassium at 30 and 240 minutes of perfusion with heterologous blood.

Table X : The mean values of platelets in heterologous perfusions using methods to prevent aggregation or the immune response.

Table X : The mean values of leucocytes in heterologous perfusions using methods to prevent aggregation or the immune response.

	Temp. at:		Resistance at:			Total flow at:
	0''	30''	0''	0''	0''	0''
Hydrocortisone	20	36	0,098	0,025	0,28	0,90
Trasylol	20	36	0,140	0,060	0,35	0,61
Periactin	20	34	0,150	0,023	0,30	0,80
Human flush	28	36	0,116	0,061	0,36	0,68
Plasma flush	20	37	0,120	0,020	0,25	1,10
Cold	20	12	0,090	0,050	0,30	0,30
Heparin	20	35	0,130	0,057	0,25	0,59
Persantin	20	36	0,105	0,050	0,26	0,89
Colloidal carbon	20	35	0,120	0,085	0,18	0,68
KRB + KG	28	38	0,075	0,032	0,58	1,17
KRB + KG + EDTA	26	38	0,089	0,032	0,37	0,99

	Platelets % PV/HV				
	1"	5"	15"	30"	180"
Hydrocortisone	89/38	75/39	68/31	43/32	-
Trasylol	98/52	68/27	29/19	26/17	-
Periactin	102/55	100/23	-	13/12	-
Human flush	107/52	110/42	66/34	57/29	-
Plasma flush	105/78	84/57	61/31	44/38	-
Cold	82/64	104/35	52/24	26/21	-
Heparin	101/55	94/19	77/14	36/11	-
Persantin	98/85	86/53	29/17	13/12	-
Colloidal carbon	76/59	88/26	27/10	16/12	-
KRB + KG	102/93	104/62	85/55	58/37	12
KRB + KG + EDTA	97/60	82/39	57/30	33/23	11
Control	86/77	83/68	36/28	29/25	-

	Asp.T/sam		Plasma Hb		Plasma K^+	
	30"	240"	30"	240"	30"	240"
Hydrocortisone	14	24	15	108	0,9	0,3
Trasylol	3	418	9	161	0,6	0,9
Periactin	112	247	8	144	0,4	0,1
Human flush	22	136	16	93	0,2	0,8
Plasma flush	170	180	16	92	1,4	1,9
Cold	65	70	5	110	0,8	4,1
Heparin	20	150	50	90	1,8	2,9
Persantin	80	170	10	100	0,1	0,2
Colloidal carbon	50	335	20	180	0,2	0,5
KRB + KG	55	100	30	84	2,7	1,7
KRB + KG + EDTA	90	195	11	75	2,5	4,0

	Leucocytes % PV / HV				
	1"	5"	15"	30"	180"
Hydrocortisone	120/50	101/54	81/37	52/45	-
Trasylol	104/64	91/47	55/40	55/45	-
Periactin	80/53	67/9	41/9	11/4	-
Human flush	104/51	107/32	78/26	59/25	-
Plasma flush	125/73	107/87	63/54	57/49	-
Cold	82/61	87/29	37/14	23/14	-
Heparin	93/49	76/30	75/16	37/15	-
Persantin	120/83	87/44	38/34	33/17	-
Colloidal carbon	83/38	78/15	34/11	11/11	-
KRB + KG	109/64	102/26	77/56	34/29	45
KRB + KG + EDTA	94/67	97/18	54/22	25/12	22

B - Attempts to prevent platelet aggregation in *heterologous* perfusion

In Table X, the same factors as described above are judged in the results of 11 groups of perfusions with human blood.

The use of Trasylol, Periactin, cold perfusion, Heparin, and Persantin did not result in any improvement in platelet and leycocyte levels. Addition of colloidal carbon not only made no difference to the cell counts, but resulted in a marked increase in portal resistance with the production of large amounts of ascites from the liver surface.

The use of Hydrocortisone, EDTA or the flushing with human blood or plasma, and the addition of αKG improved the fall in cell counts a little, but no major improvement was noted.

No difference was made by any of these techniques to the presence of antibodies, nor to the decrease in complement levels.

Interpretation:

As a result of the experiments, it was concluded that the pig liver could not be used again for clinical perfusion with safety before a means was available of removal of antibody with associated improvement in trans-hepatic platelet differences. The use of forced flow electrophoresis in the separation of antibody has been shown to result in prolongation of heterograft survival, and could be of great value. However, at present it remains an experimental tool and requires full investigation before more general use.

Another possible means of avoiding the thrombocytopenia lay in the choice of another animal for donation of the liver. Experiments in this direction are described in chapter Eight.

CHAPTER EIGHT

The use of other animal livers

Introduction

Phase VIII

(i) Design:

1) Animals

(a) Calf with human blood

(b) Lamb with human blood

(c) Baboon with compatible human blood

(d) (i) Baboon with human blood of Group O

(ii) Baboon with incompatible human blood

2) Other conditions

(ii) Results:

A

Interpretation

B

Interpretation

C

Interpretation

D (i)
(ii)

Interpretation

Introduction:

Livers from calves had been used for clinical assist purposes (50) and sheep livers had been used for physiological studies (156). No haematological investigations were performed in either of these two groups of experiments. Since calves and sheep were readily available it was decided to investigate their use and the effect upon platelet counts of perfusion of these livers with human blood. Although the relationship of the heterograft response under review to all pre-formed antibodies is not completely defined, the observation that human blood appeared to possess a lower titre of heterophile antibodies against sheep cells, also encouraged the use of this animal for experiments.

The baboon had been used in this Unit (235) and elsewhere (125) as a cross circulation partner for patients in hepatic coma. In other centres though, the animal has not been used for experiments due to its unavailability. The use of the baboon was investigated in this study especially because of the similarity of the blood group substances which are secreted by these animals, to those carried on human cells. In addition, heterophile antibodies had not been detected in human blood against baboon erythrocytes.

PHASE VIII

(i) **Design:**

1) Animals

(a) Three livers from young calves were perfused for four hours with human blood of random groups.

(b) The livers from three lambs were perfused with human blood of random groups.

(c) Four baboon livers were perfused with compatible human blood for four hours.

(d) In regard to the clinical application of this procedure it was important to investigate the necessity of patient cross matching with the animal. Since no baboon is grouped as human group O, livers from baboons of groups A, B or AB were perfused with human blood of group O to assess the effect of the anti-A and anti-B antibodies present.

(i) The liver from a baboon of group B was perfused with group A human blood and

(ii) one of group AB with group A human blood.

Since group B human blood was very scarce, perfusion of a group A baboon liver and human group B was not performed.

2) Other conditions

— In the above experiments, the conditions used were those which had given the best results

in previous work. These included flushing the liver with Krebs Ringer bicarbonate at 38°C, using 1,75 litres through the portal vein and 0,25 litres through the hepatic artery. The circuit was primed with 0,25 litres of McCoys medium 5A and 1,0 litres of human blood. A constant infusion of the hormonal "cocktail" was used, and 15 mmol αKG wa added per litre to the flushing solution, the prime and the infusion.

— The diaphragm was kept mobile.

— The conditions of dissection were different in these animals in that in each case, the anatomy was variable and unfamiliar. Although this was not thought to add to the trauma of removal of the liver, the duration of operation was prolonged. In the case of the baboon liver dissections, the animals used were very small which complicated the procedure. In addition, since no change was made in the volume of the flushing solution, these livers were flushed to a relatively greater degree than the larger animal livers.

(ii) **Results:**

A: The results of perfusion of the calf liver with human blood are shown in Figures 33-36.

— Physical and biochemical results:

Portal resistance was within accepted levels but the total flow rate was much less than the arbitrary maximum. Bile flow was slow, especially in view of the large size of the liver, and after two hours, frank haemobilia was seen in all three experiments (Figure 33).

The mean levels of aspartate transaminase were no higher than those noted in perfusions of pig liver with human blood, but plasma haemoglobin levels were higher (Figure 34).

The clearance of the ammonia load was less efficient than that in perfusions of the pig liver. The lactate/pyruvate ratios were much higher throughout perfusion; the increase in plasma urea was similar to the results obtained in perfusions of the pig liver with human blood (Figure 35).

— Haematological and immunological

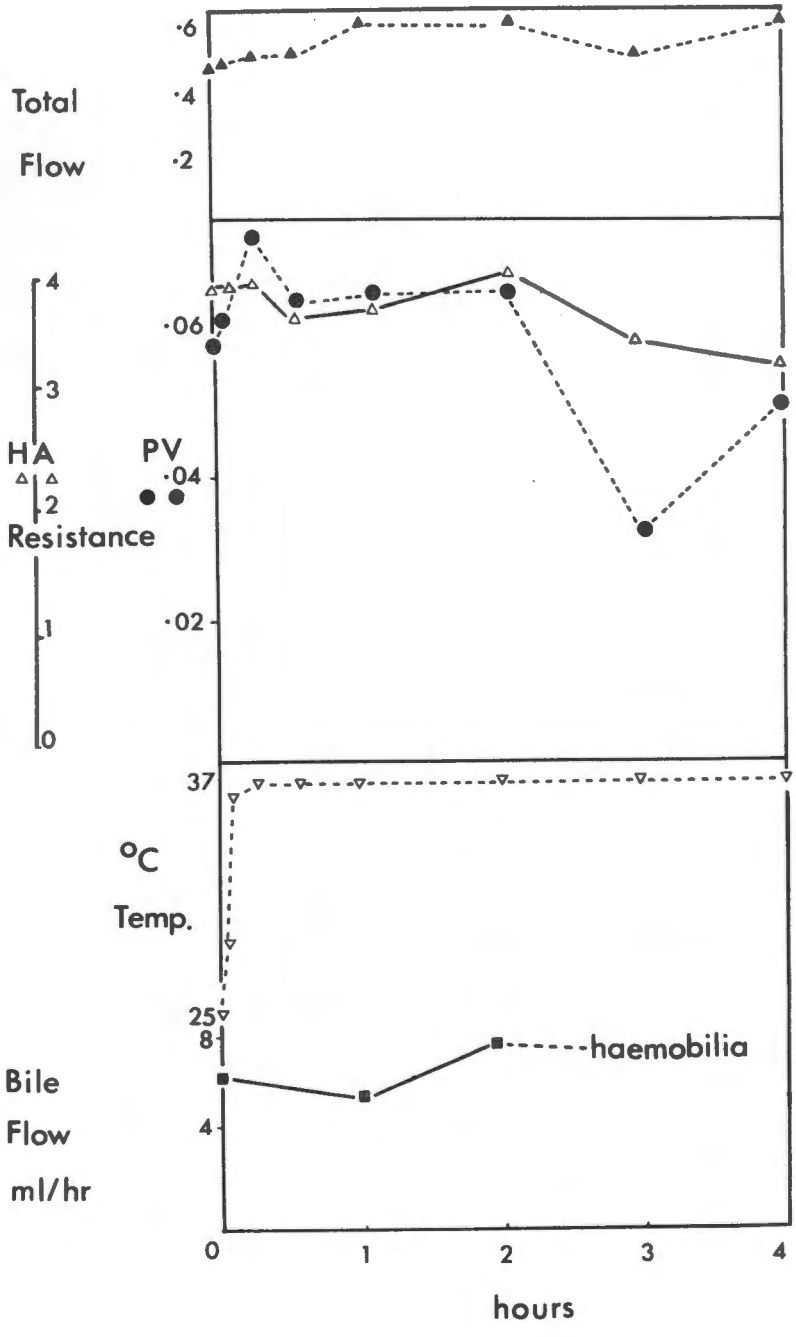
Platelet counts (Figure 36) followed the same pattern as in perfusions of the pig liver with human blood. Complement levels decreased by a mean value of 47% within the first 30 minutes of perfusion. The measurement of antibodies in human blood against calf cells was complicated by the inability to prepare lymphocytes and leucocytes by the methods used for human and porcine cells. It was found impossible to remove erythrocytes from calf blood, which also required longer for defibrination.

Interpretation:

No improvement in any factor resulted from the use of the calf liver. In fact, additional

Figure 33 : The mean values of portal resistance, total flow and bile flow in perfusion of the calf liver with human blood.

(The inset in all subsequent figures shows the comparative values in perfusions of the pig liver with human blood).


Total
Flow
·6
·4
·2
HA
PV
Resistance
4
3
2
1
0
·06
·04
·02
37
°C
Temp.
25
8
4
haemobilia
Bile
Flow
ml/hr
0
1
2
3
4
hours

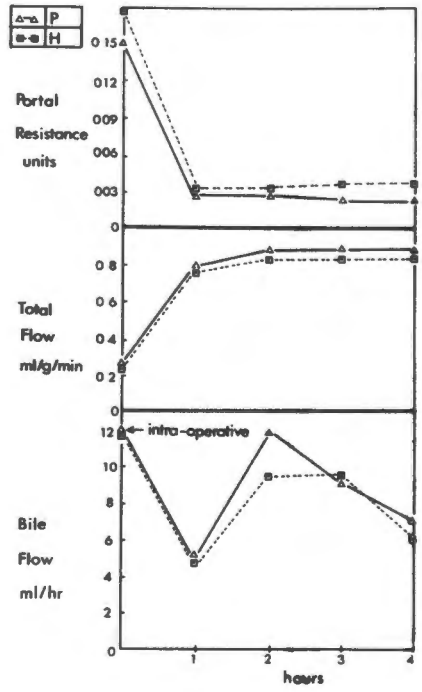
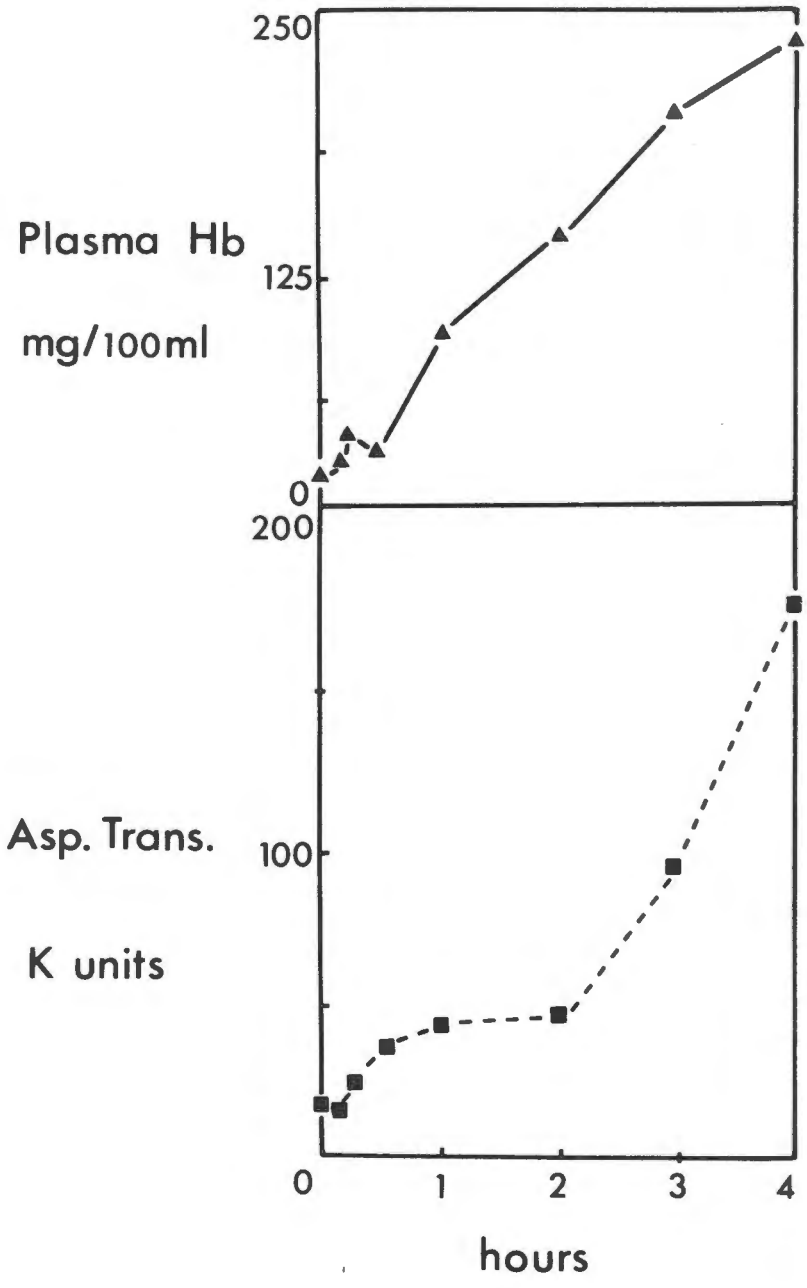

P
H
Portal
Resistance
units
0 15
012
009
006
003
0
Total
Flow
ml/g/min
0 8
0 6
0 4
0 2
0
intra-operative
12
10
8
6
4
2
0
Bile
Flow
ml/hr
1
2
3
4
hours

Figure 34 : The mean levels of aspartate transaminase and plasma haemoglobin in perfusions of the calf liver with human blood.


Plasma Hb
mg/100ml
250
125
0
200
Asp. Trans.
K units
100
0
1
2
3
4
hours

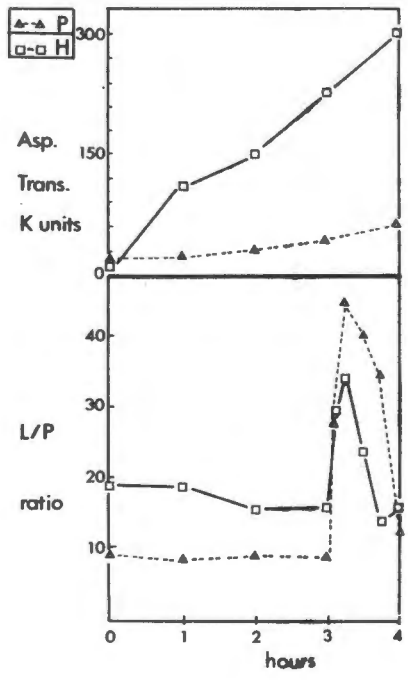
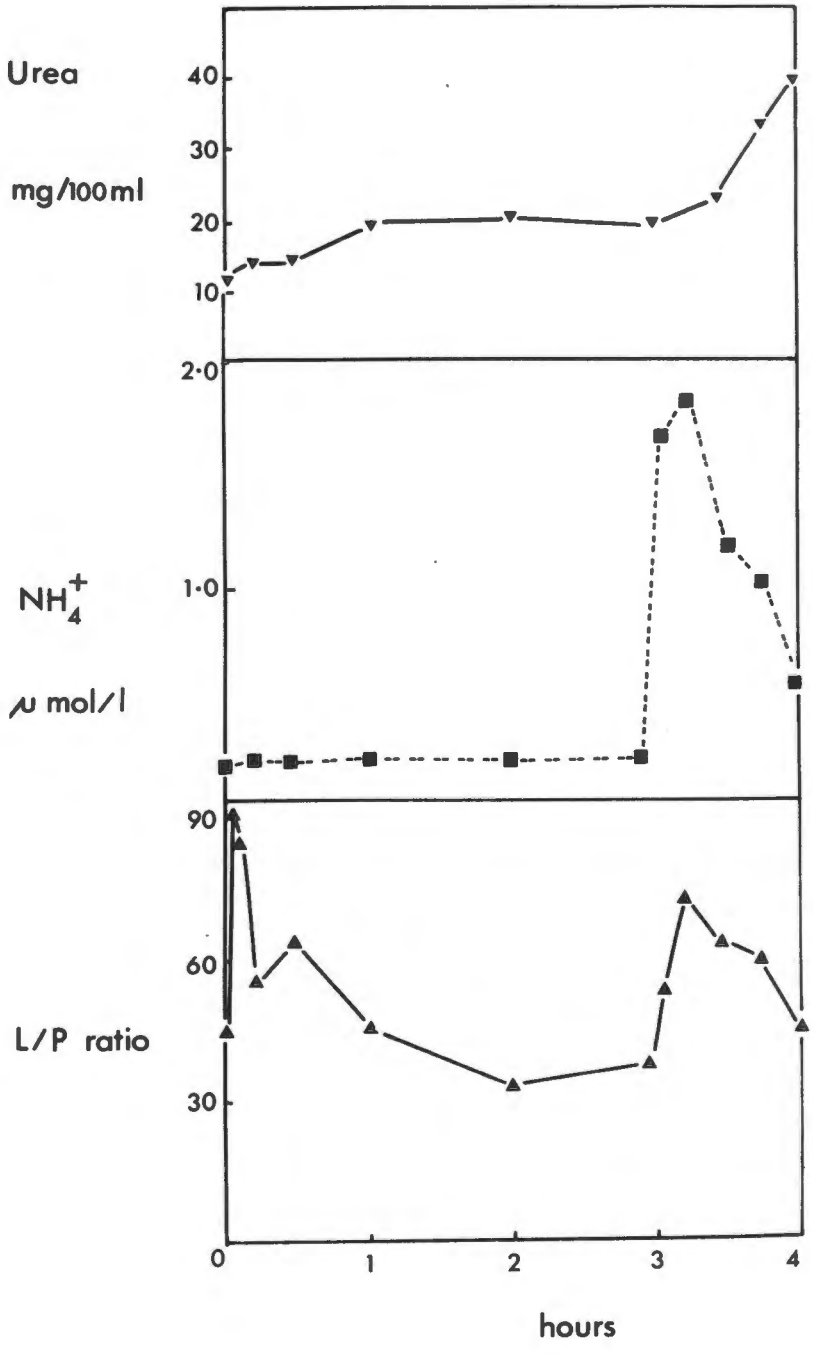
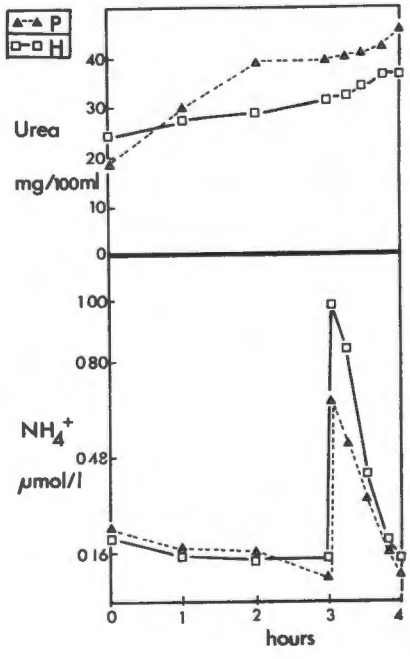

P
H
300
Asp.
Trans.
K units
150
0
40
30
L/P
ratio
20
10
0
1
2
3
4
hours

Figure 35 : The mean levels of ammonia and lactate/pyruvate ratios in perfusions of the calf liver with human blood.


Urea
mg/100ml
40
30
20
10
2·0
1·0
NH_4^+
μ mol/l
90
60
30
L/P ratio
0
1
2
3
4
hours


P
H
Urea
mg/100ml
40
30
20
10
0
100
080
NH_4^+
μmol/l
048
016
0
1
2
3
4
hours

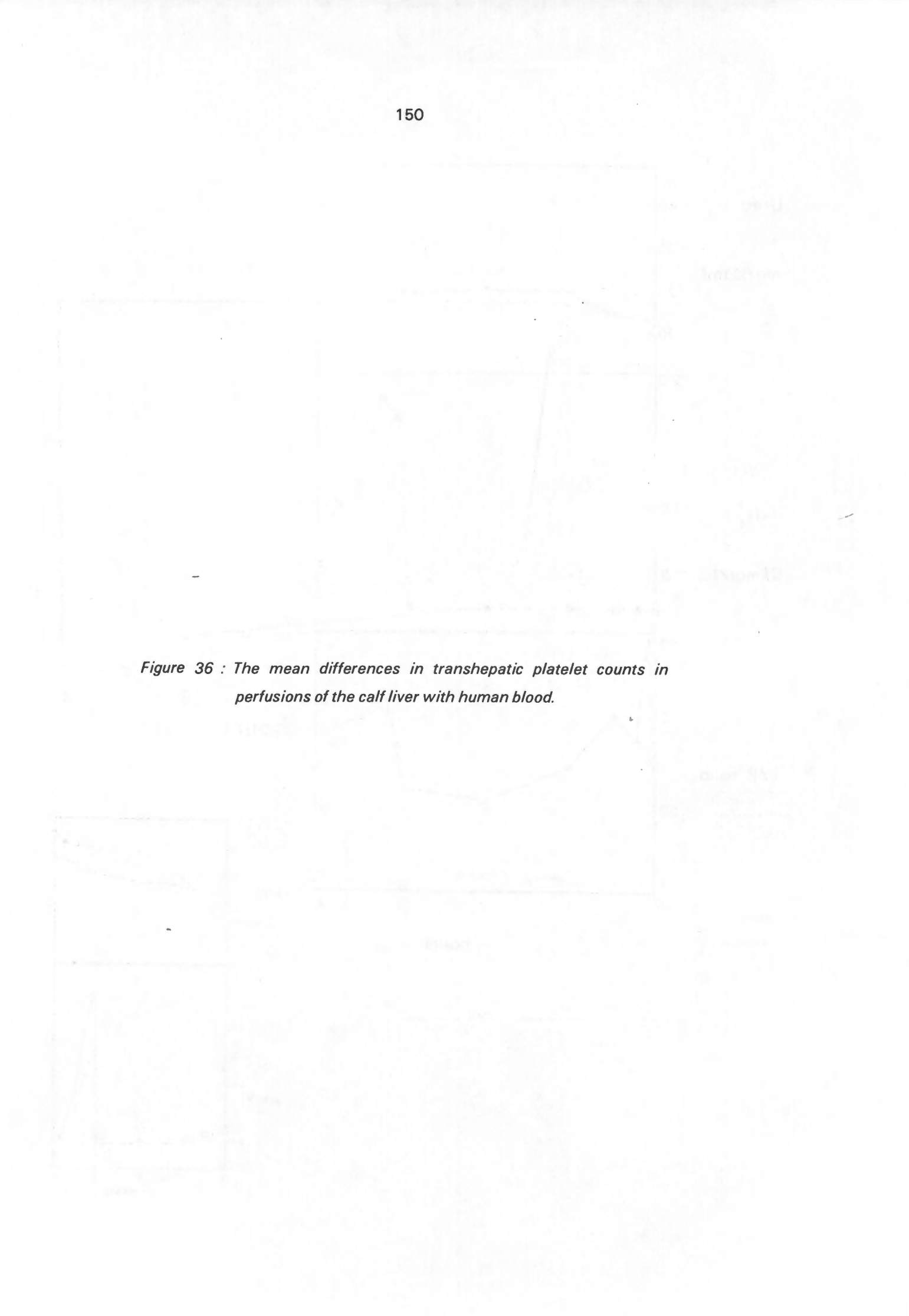
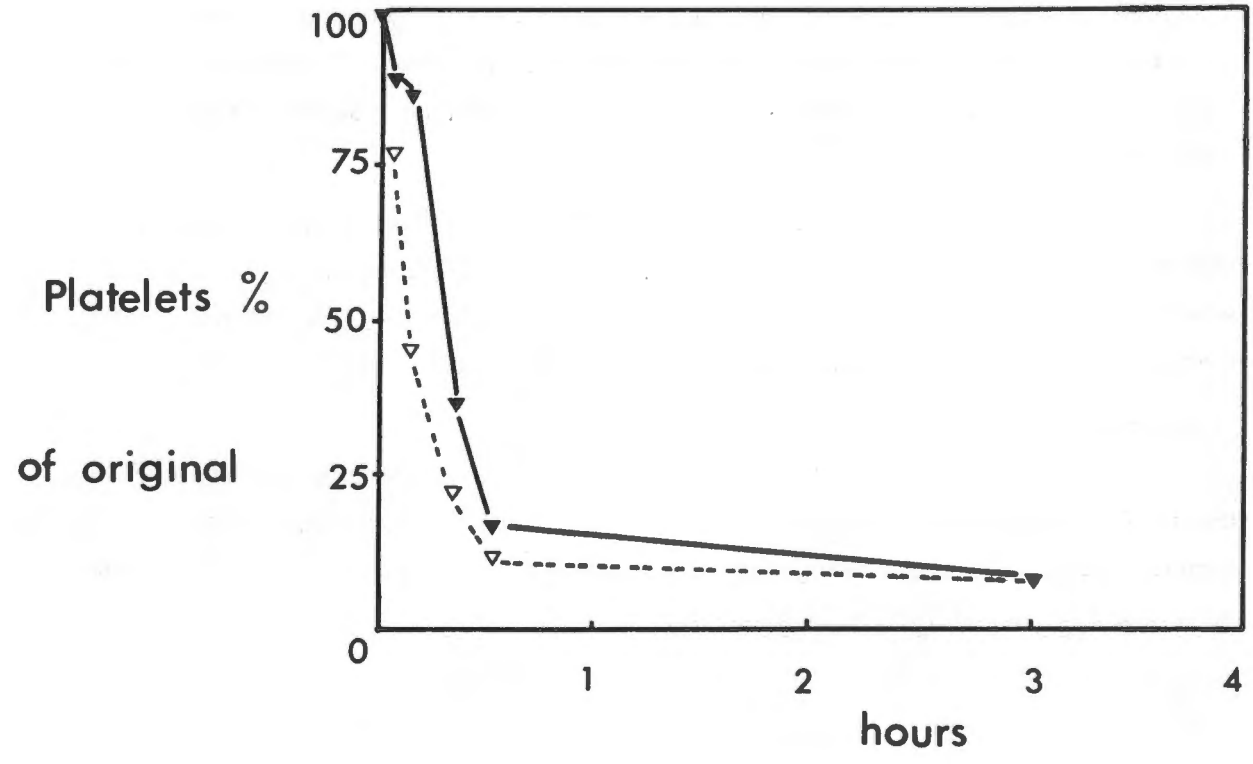
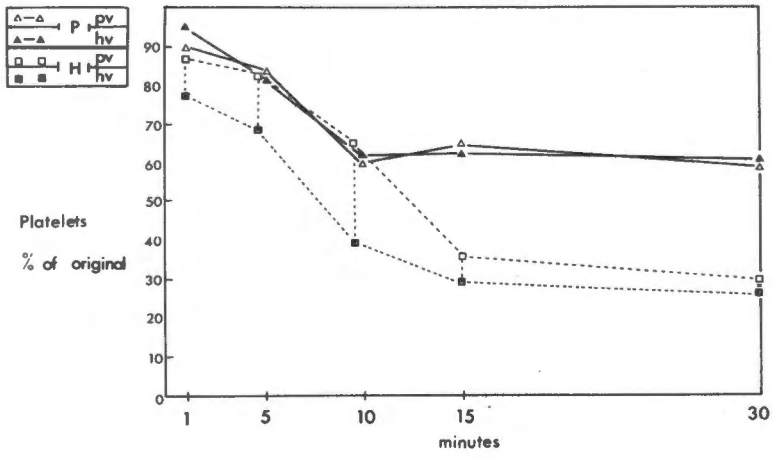


Figure 36 : The mean differences in transhepatic platelet counts in perfusions of the calf liver with human blood.


Platelets %
of original
100
75
50
25
0
1
2
3
4
hours


P pv hv
H pv hv
Platelets
% of original
90
80
70
60
50
40
30
20
10
0
1
5
10
15
30
minutes

damage appeared to result from this form of perfusion, although no comparative studies have been made with bovine blood.

B: The results of perfusion of the lamb liver with human blood are shown in Figures 37-40.

— Physical and biochemical results:

Portal resistance which was initially high, fell during the first 15 minutes to lower levels than those recorded during heterologous porcine liver perfusion. The hepatic arterial resistance was also lower, and the total flow rate was a little lower than the arbitrary maximum. Bile flow was rapid and sustained by the choleretic "cocktail".

The mean levels of aspartate transaminase were higher than those noted in heterologous porcine liver perfusion although the plasma haemoglobin levels were similar (Figure 38). There was a rapid clearance of the ammonia and the lactate loads, and urea production was greater than was noted in heterologous porcine liver perfusions (Figure 39).

— Haematology and immunology:

Platelet counts showed a similar marked decline during the first thirty minutes of perfusion (Figure 40). Complement levels decreased by 40% within the first 30 minutes of perfusion and lymphocytotoxic, leucoagglutinating and heterophile antibodies were reduced within five minutes and almost totally absent at 30 minutes.

Interpretation:

Thus the lamb liver showed no obvious improvement in the fall in platelets. Other physical and biochemical aspects of these experiments gave similar results to heterologous perfusion of the calf or pig liver.

C: Perfusion of the baboon liver with compatible blood:

The results of these experiments are shown in Figures 41-44.

— Physical and biochemical results:

It appears that the arbitrary maximum total flow could be more rapidly achieved within thirty minutes without excessive rise in portal vascular resistance (Figure 41). The hepatic arterial pressure was, however, higher than was encountered in other perfusions. This could perhaps be related to the fact that the hepatic arterial system could not be directly cannulated and an aortic segment had to be used. During this process, the possibility of air embolus was greater, but in other perfusions, even if air embolus occurred, the resultant increase in resistance resolved within a short period. The arterial resistance in this series of experiments remained higher than recommended by others (61). The bile flow obtained in all periods of perfusion was less than with other livers despite the use of the choleretic solution. It was noted that the bile production was less during the operation also, and that the bile was dark green

and more viscid than pig bile.

Figure 42 shows the levels of aspartate transaminase and plasma haemoglobin in perfusions of the baboon liver with compatible human blood compared with levels during homologous or heterologous perfusion of the pig liver. There was a similar rise in aspartate transaminase to that occurring in homologous perfusion, but the increase in plasma haemoglobin was greater in perfusions of the baboon liver.

The clearances of ammonia and lactate, appeared similar to those in other experiments. (Figure 43).

— Haematological and Immunological:

The results of the transhepatic platelet study (Figure 44) reveal that there is a marked difference in the reduction in platelet counts across the liver. The initial difference of 30% at one minute reduces to 10% at 5, 15 and 30 minutes, seventy seven per cent of the original platelet level remains at three hours. This level is statistically highly significant when compared with results obtained when pig livers were perfused with human blood ($p<0,001$).

Mean total complement levels declined 12% over the course of the first thirty minutes of perfusion - (cf. in perfusions of the pig liver with human blood, the decline was 28% which is significantly different - $p<0,001$).

Lymphocytotoxic and leucoagglutinating antibodies were not detected in human blood at any stage, and heterophile antibodies against guinea pig, rat, and pig were not reduced.

Interpretation:

The use of the baboon liver seemed therefore, to provide the first hope of an improvement in the trans-hepatic decrease in platelets. In regard to the other functions measured, these seemed to be performed in a manner similar to the homologous perfusions of the pig liver with pig blood.

The question which remained still to be answered concerned the necessity for cross-matching of the animal and the human blood used.

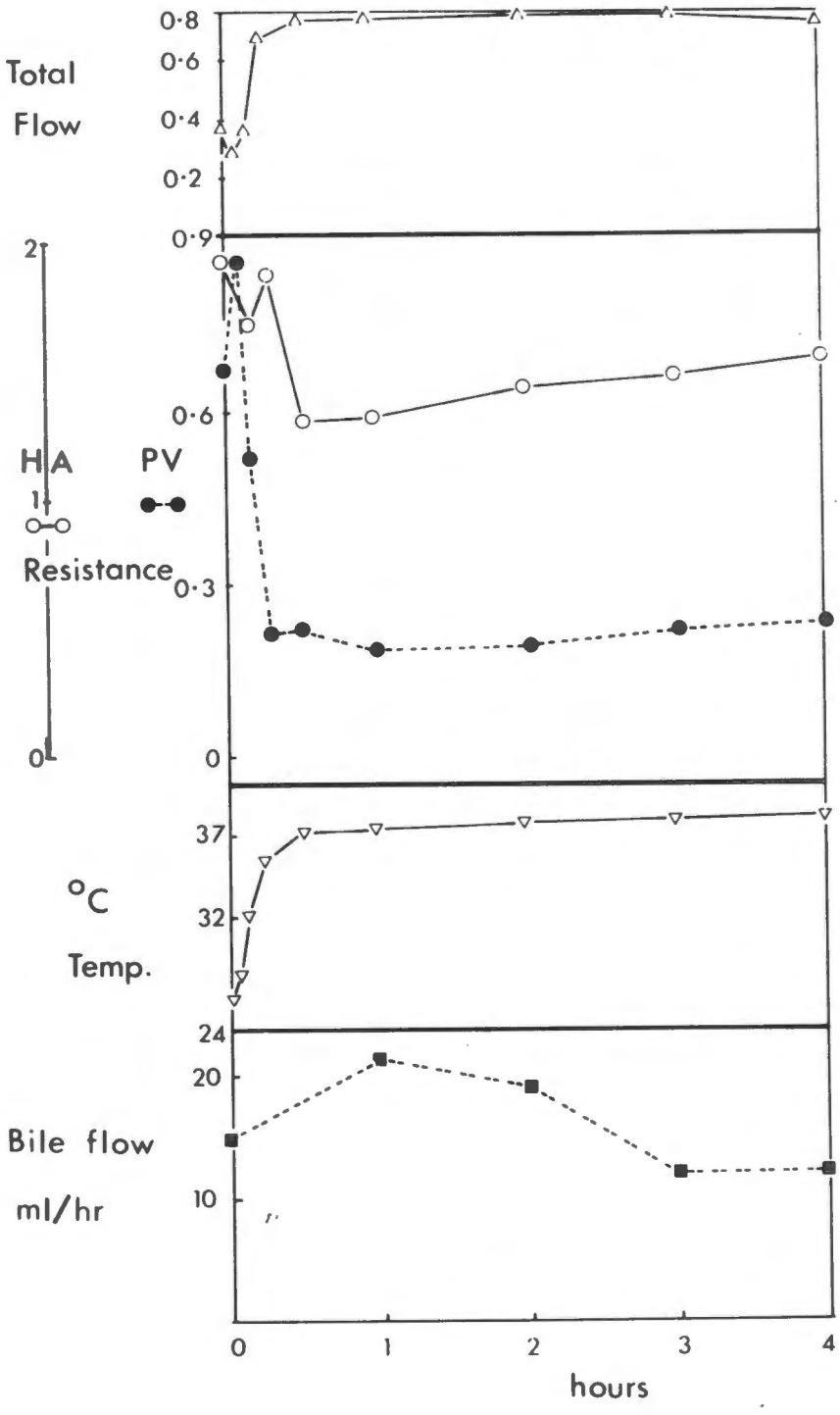
D: The results of

(i) 1 perfusion of the liver from a baboon group A with human blood of group O low titre. (2 hours).

(ii) 1 perfusion of the liver from a baboon group A with human blood of group O high titre. (4 hours).

(iii) 1 perfusion of the liver from a baboon group B with human blood of group A. (1 hour).

Figure 37 : The mean values of portal resistance, total flow and bile flow in perfusions of the lamb liver with human blood.


Total
Flow
0·8
0·6
0·4
0·2
0·9
2
HA
PV
1
Resistance
0·6
0·3
0
37
°C
32
Temp.
24
20
Bile flow
ml/hr
10
0
1
2
3
4
hours

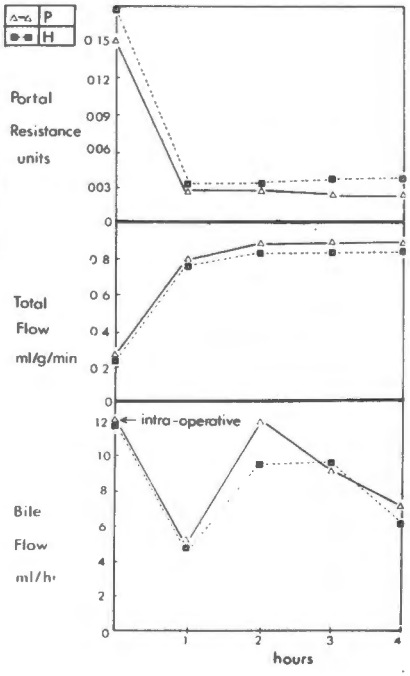
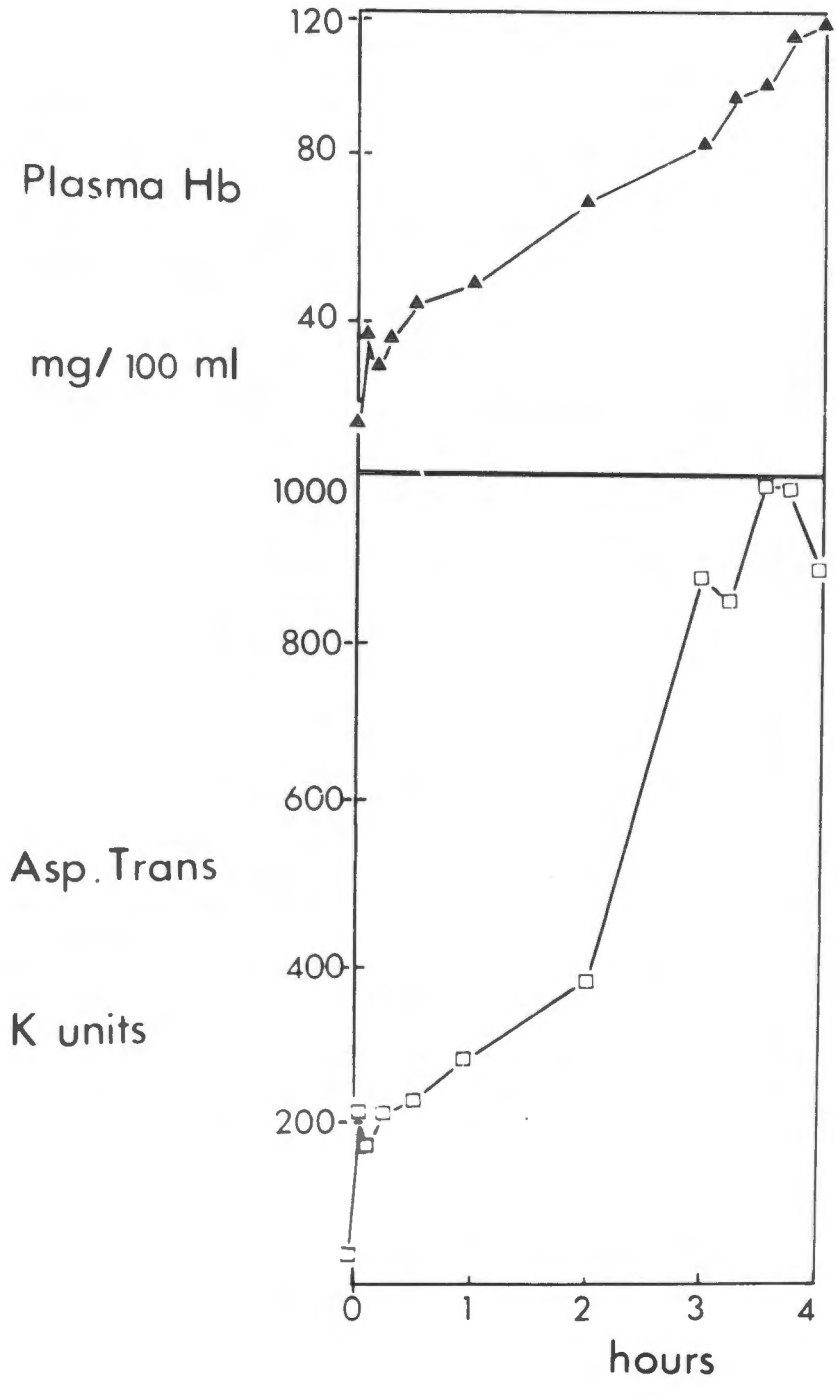

P
H
Portal
Resistance
units
0·15
0·12
0·09
0·06
0·03
0
Total
Flow
ml/g/min
0·8
0·6
0·4
0·2
0
intra-operative
Bile
Flow
ml/hr
12
10
8
6
4
2
0
1
2
3
4
hours

Figure 38 : The mean levels of aspartate transaminase and plasma haemoglobin in perfusions of the lamb liver with human blood


Plasma Hb
mg/ 100 ml
120
80
40
1000
800
600
Asp. Trans
K units
400
200
0
1
2
3
4
hours

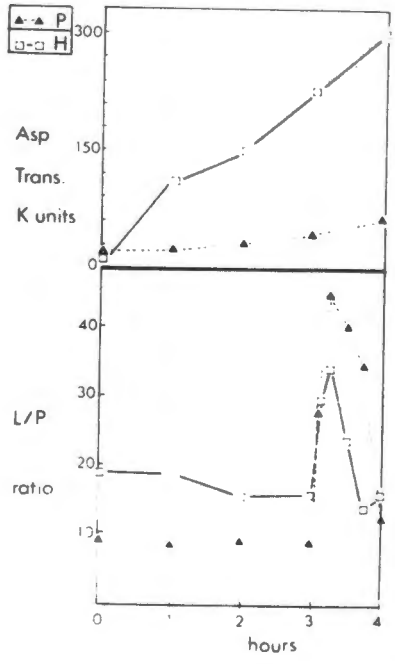
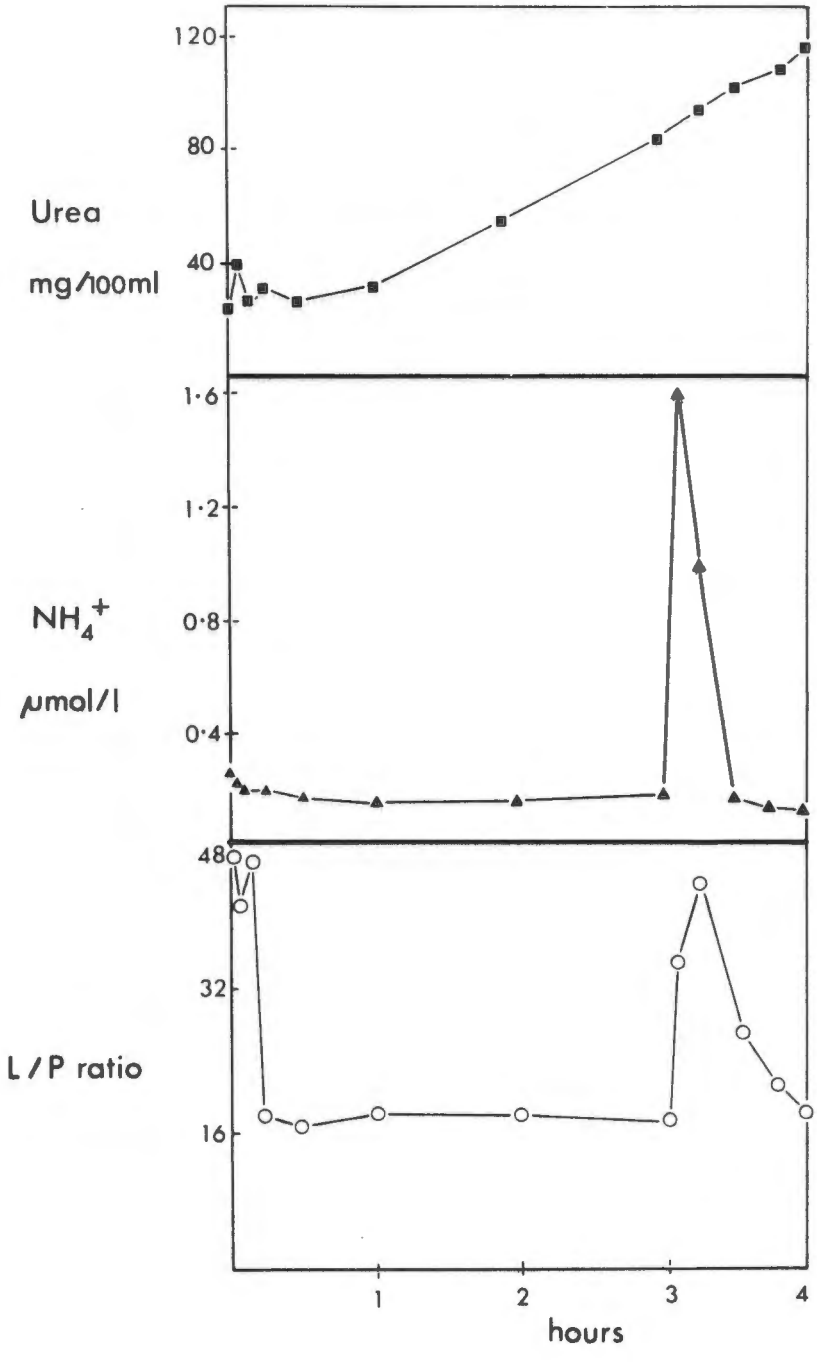

P
H
300
Asp
150
Trans.
K units
0
40
30
L/P
20
ratio
10
0
2
3
4
hours

Figure 39 : The mean levels of ammonia and lactate/pyruvate ratios in perfusions of the lamb liver with human blood.


120
80
40
Urea
mg/100ml
1·6
1·2
0·8
0·4
NH_4^+
µmol/l
48
32
16
L/P ratio
1
2
3
4
hours

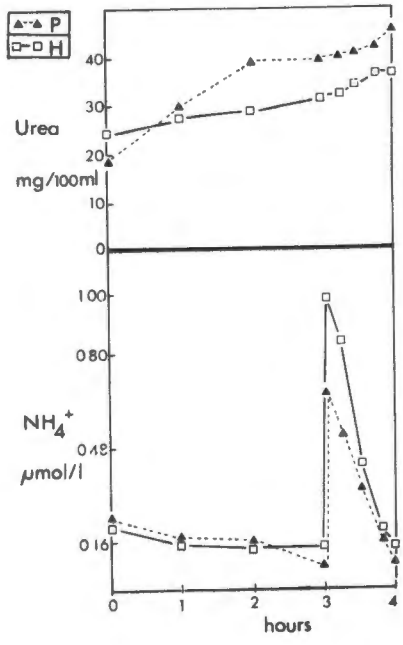
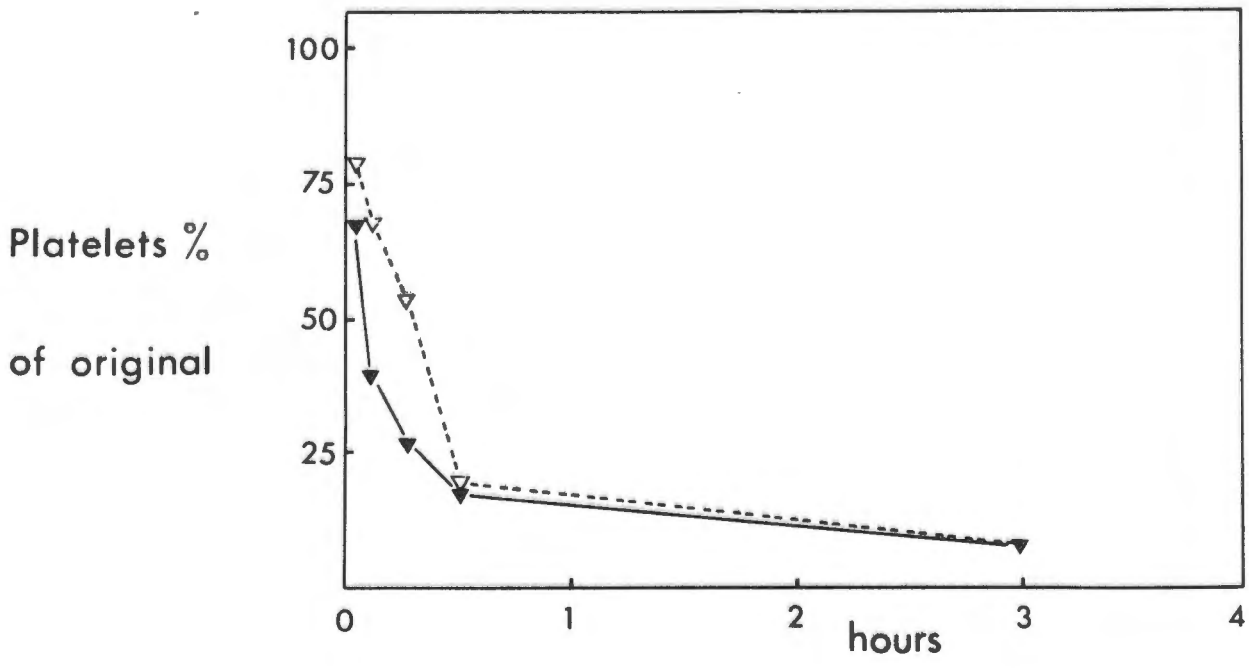

P
H
40
30
20
Urea
mg/100ml
10
0
100
080
NH_4^+
µmol/l
016
0
1
2
3
4
hours

Figure 40 : The mean differences in transhepatic platelet counts in perfusions of the lamb liver with human blood.


Platelets %
of original
100
75
50
25
0
1
2
3
4
hours

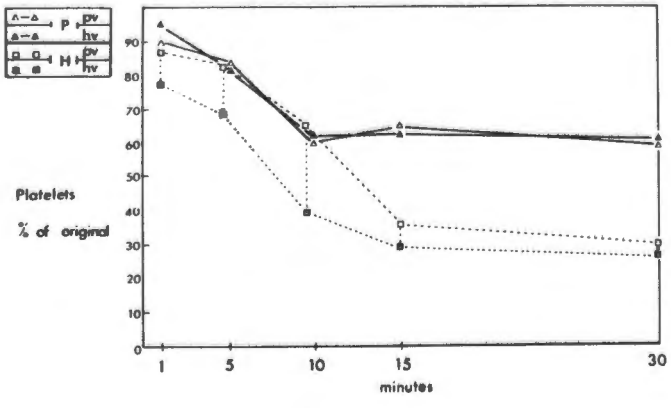

P pv
hv
H pv
hv
Platelets
% of original
90
80
70
60
50
40
30
20
10
0
1
5
10
15
30
minutes

Figure 41 : The mean values of portal resistance, total flow and bile flow in perfusions of the baboon liver with human blood.

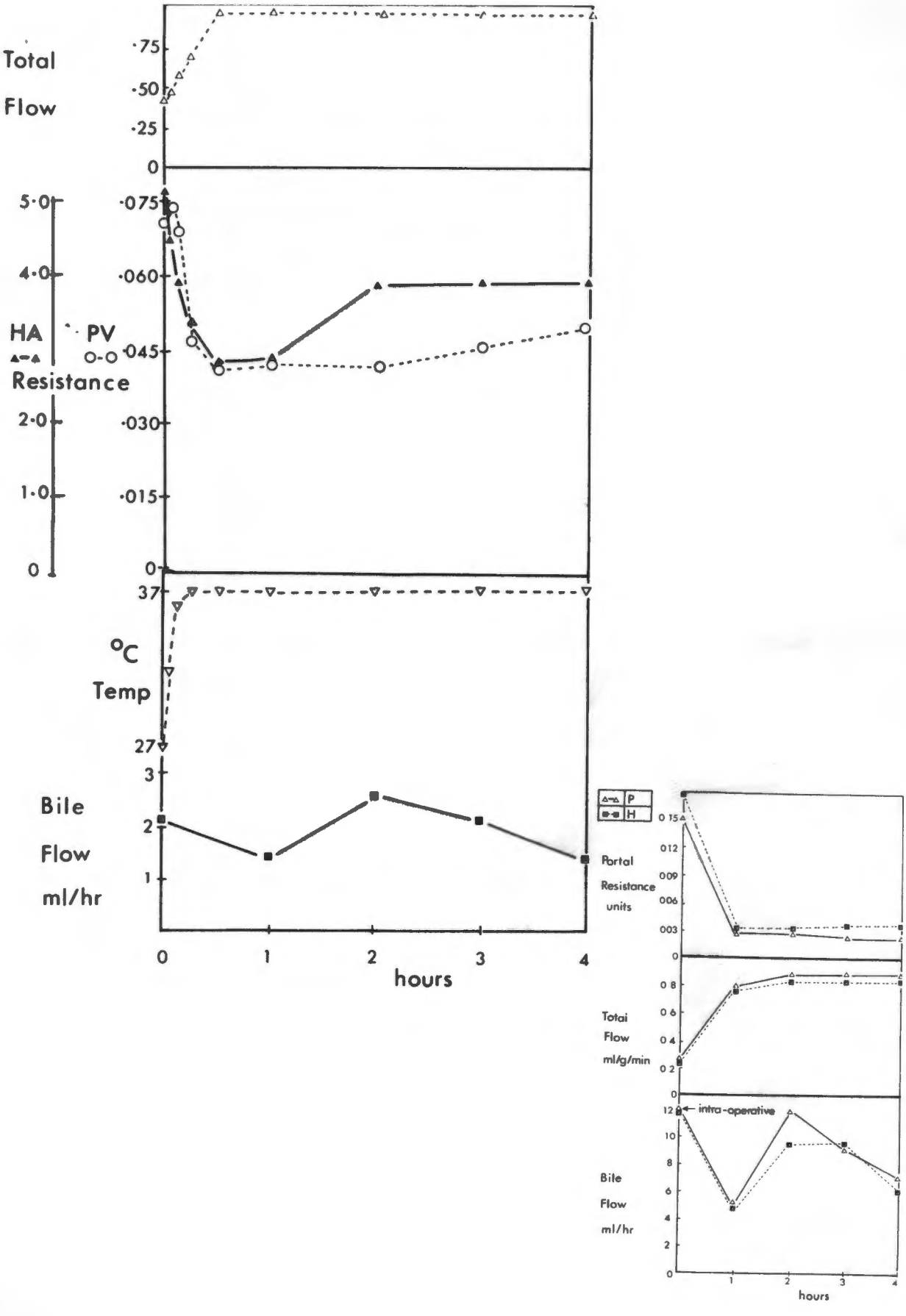
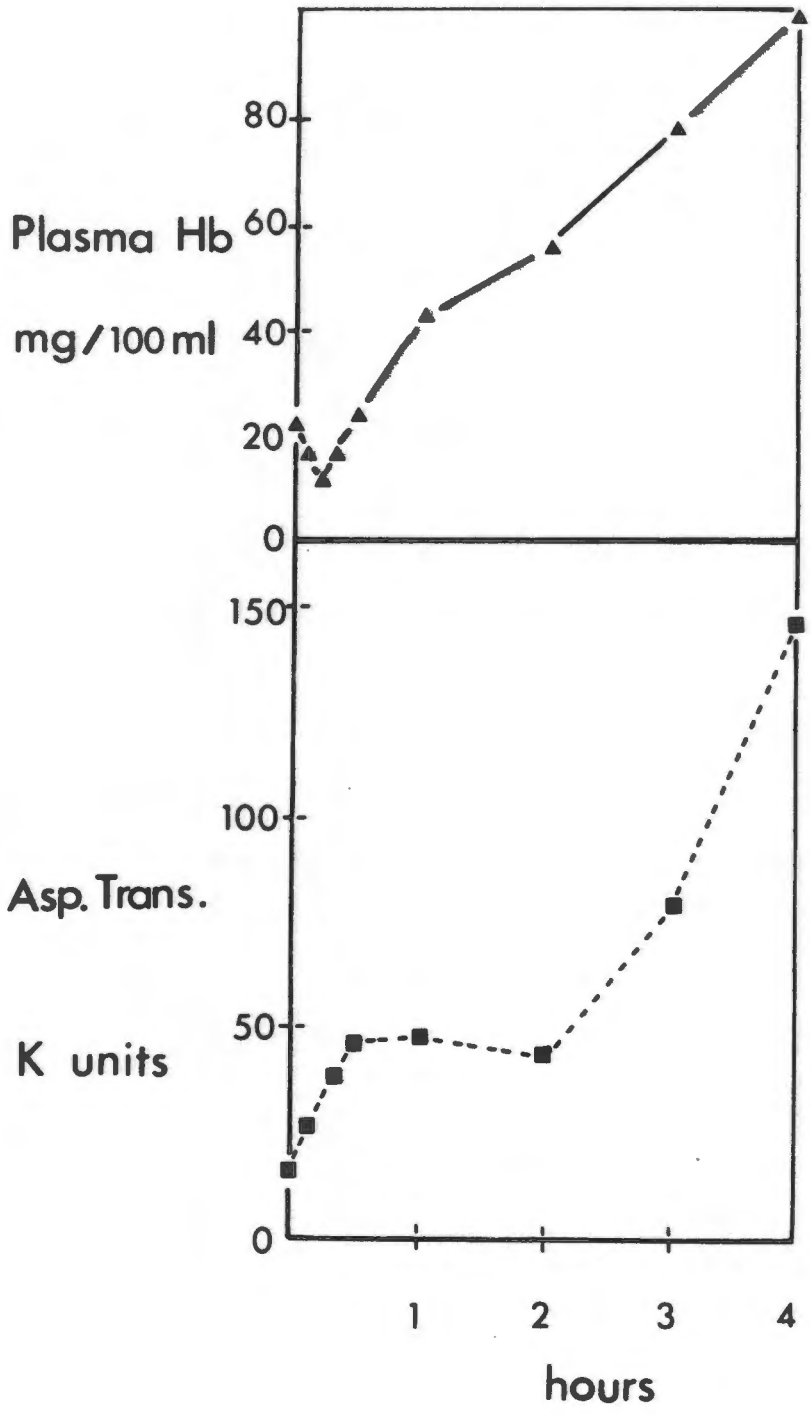

Total
Flow
·75
·50
·25
0
5·0
4·0
HA
PV
Resistance
2·0
1·0
0
·075
·060
·045
·030
·015
0
37
°C
Temp
27
3
Bile
Flow
ml/hr
2
1
0
1
2
3
4
hours
P
H
Portal
Resistance
units
0·15
0·12
0·09
0·06
0·03
0
0·8
0·6
0·4
0·2
0
Total
Flow
ml/g/min
12
10
8
6
4
2
0
← intra-operative
Bile
Flow
ml/hr
1
2
3
4
hours

Figure 42 : The mean levels of aspartate transaminase and plasma haemoglobin in perfusions of the baboon liver with human blood.


Plasma Hb
mg/100 ml
80
60
40
20
0
150
100
Asp. Trans.
K units
50
0
1
2
3
4
hours

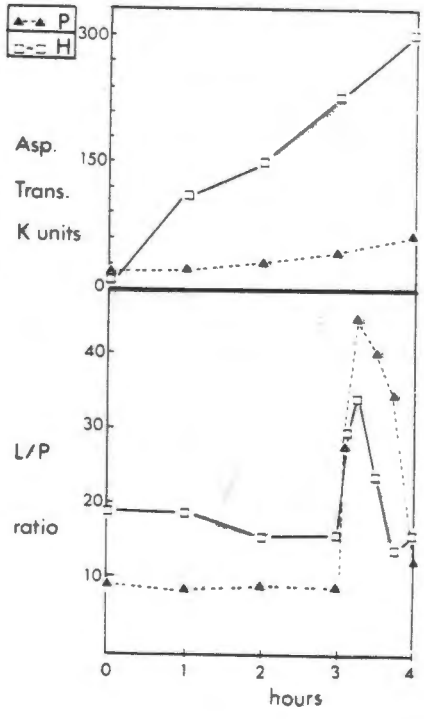
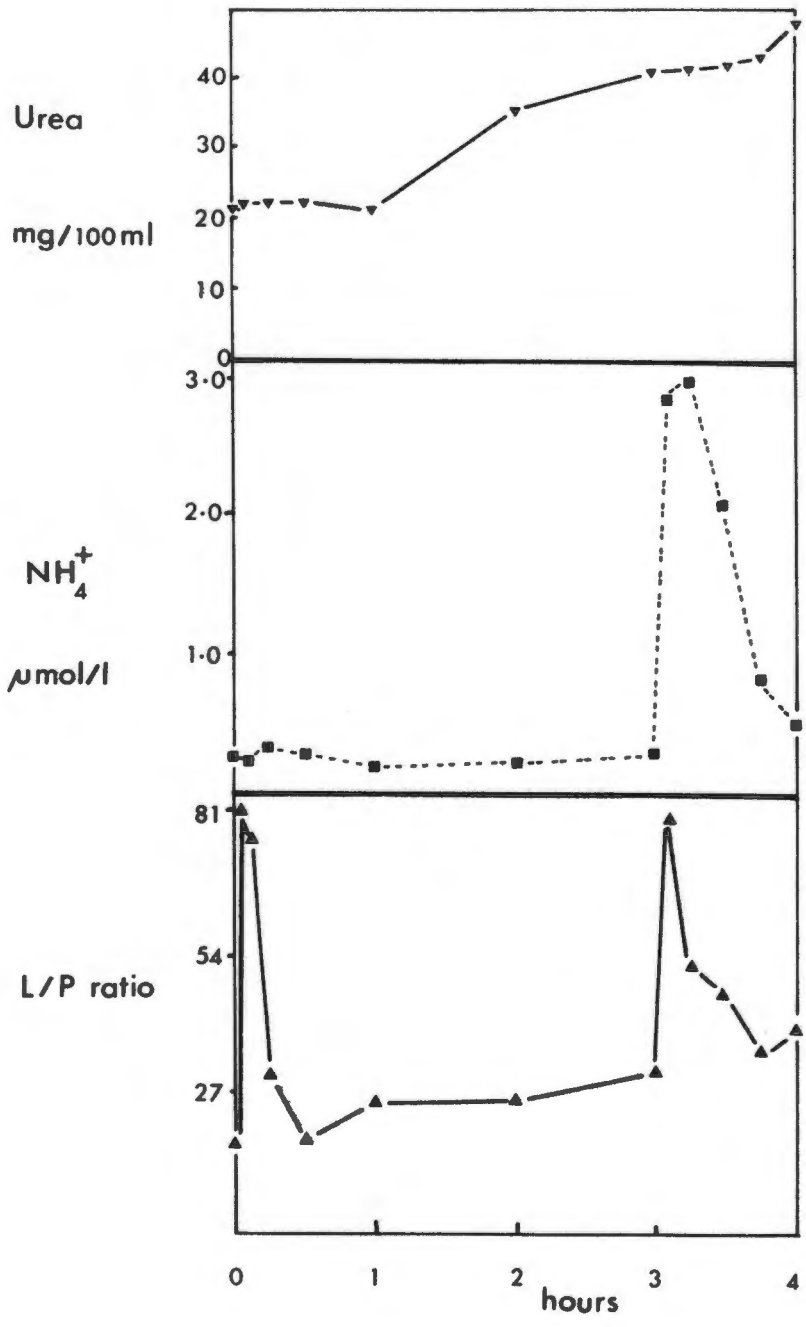
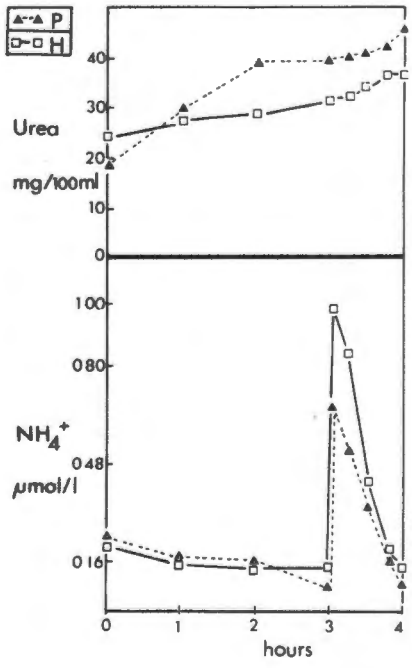

P
H
300
150
Asp.
Trans.
K units
0
40
30
L/P
ratio
20
10
0
1
2
3
4
hours

Figure 43 : The mean levels of ammonia and lactate/pyruvate ratios in perfusions of the baboon liver with human blood.


Urea
mg/100ml
40
30
20
10
0
3·0
2·0
1·0
NH_4^+
μmol/l
81
54
27
L/P ratio
0
1
2
3
4
hours


P
H
Urea
mg/100ml
40
30
20
10
0
100
080
048
016
NH_4^+
μmol/l
0
1
2
3
4
hours

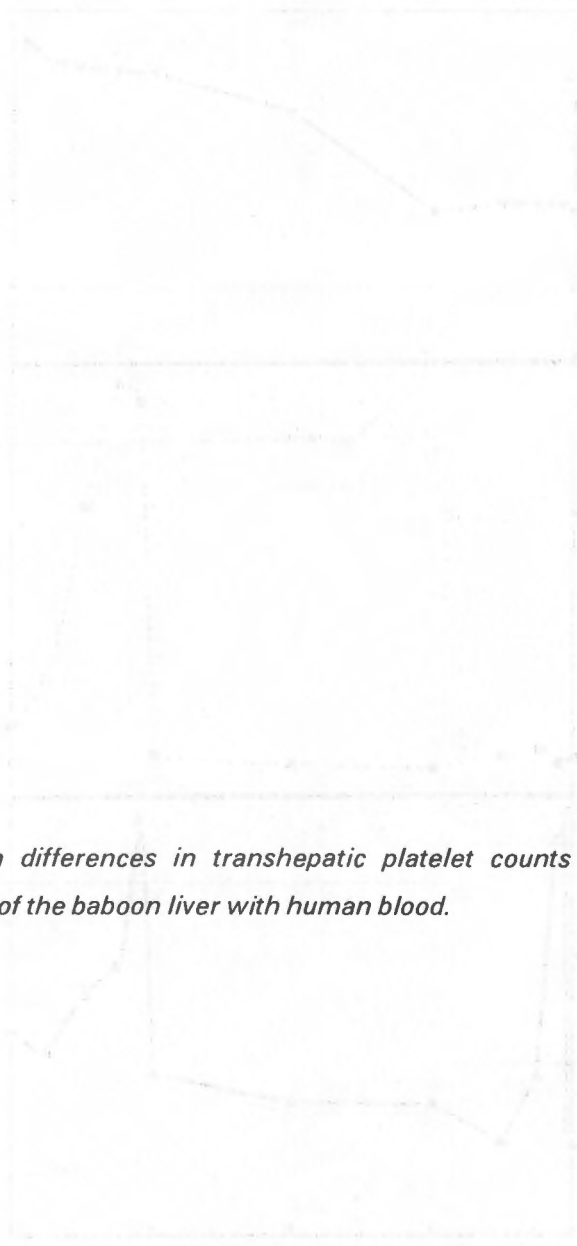
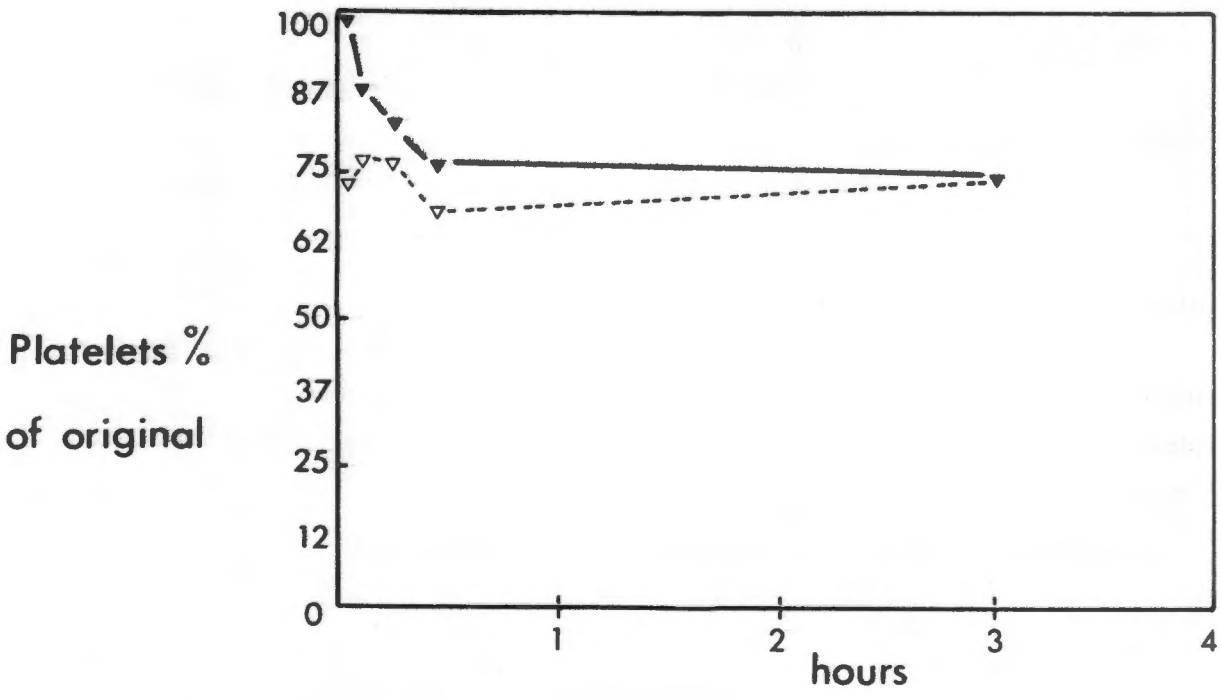
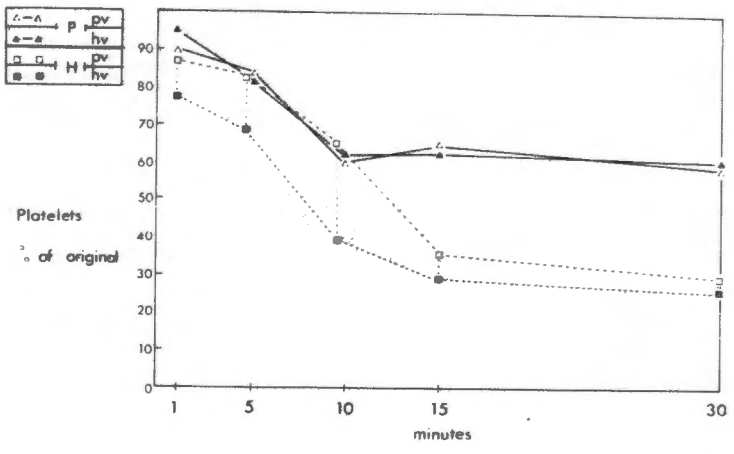


Figure 44 : The mean differences in transhepatic platelet counts in perfusions of the baboon liver with human blood.


Platelets %
of original
100
87
75
62
50
37
25
12
0
1
2
3
4
hours


P
pv
hv
H
pv
hv
Platelets
% of original
90
80
70
60
50
40
30
20
10
0
1
5
10
15
30
minutes

— Physical and biochemical results:

These are shown in Table XI. It may be seen that values for portal resistance, total flow and bile flow were similar to those in section C using compatible blood. The levels of aspartate transaminase and plasma haemoglobin and potassium appeared to be higher in these experiments than in those of section B. The clearance of the load of ammonia and lactate as completed in one experiment (ii) was similar to the result in perfusions of section B.

— Haematological and Immunological:

From Table XII it may be seen that there was no difference in the trans-hepatic decline in platelets when incompatible blood was used. Complement levels declined by 15% in (i); 11% in (ii) and 12% in (iii). No lymphocytotoxic or leucoagglutinating antibodies were detected, and there was no reduction in heterophile antibodies during perfusion.

Interpretation:

The conclusion drawn from the platelet counts of these experiments was that there was no difference whether compatible or incompatible blood was used, except perhaps in the slightly greater damage to liver cells and erythrocytes as shown by greater SGOT and plasma haemoglobin. This was not a highly significant difference. (p=0,05).

The final conclusion drawn was that cross matching of the animal with the blood group of a patient with liver failure in whom a hepatic assist was to be performed, was not necessary.

Table XI : The total flow rates, portal resistance and bile flow rates in perfusions of the baboon liver with incompatible blood.

Table XI : The levels of plasma potassium, haemoglobin and aspartate transaminase in perfusions of the baboon liver with incompatible blood.

Total flow rates: ml/g/min.

	0''	1''	5''	15''	30''	1'	2'	3'	3¼'	3½'	3¾'	4'
(i)	1,1	1,1	1,1	1,0								
(ii)	0,6	0,6	0,9									
(iii)	1,0	1,0	1,0	1,0	1,0	1,0						

Portal resistance: units

(i)	,060	,057	,066	,074	,064	,066	,067
(ii)	,040	,040	,041	,032	,032		
(iii)	,066	,066	,066	,030	,030	,066	

Bile flow ml/hr

	0	1	2	3	4
(i)	1	1	12	-	-
(ii)	3	15	23	9	11
(iii)	2,5	3,5	-	-	-

Potassium: mEq/1

	0''	1''	5''	15''	30''	1'	2'	3'	3¼'	3½'	3¾'	4'
(i)	5,2	8,6	7,1	5,1	3,6	3,2	6,1	10,4	10,7	12,6	14,2	15,7
(ii)	6,0	8,4	7,1	6,1	5,1	5,3	7,2	8,4	9,9	10,4	10,0	10,4
(iii)	5,5	8,5	7,2	5,9	4,6	4,2						

Haemcglobin: mg/100ml

(i)	10		22	30	24	39	52					
(ii)	19	17	13	8	21	17	37	56	75	83	98	95
(iii)	16	16	15	14	18	21						

Aspartate Transaminase: Karmen units

(i)	15	55	50	50	70	110	235	350	440	330	325	300
(ii)	15	20	15	20	30	30	80	115	140	160	150	200
(iii)	10	-	30	25	30	20	-	-	-	-	-	-

Table XII : The levels of blood ammonia and urea, and the lactate/pyruvate ratios in perfusions of the baboon liver with incompatible blood.

Table XII : The levels of platelets and leucocytes recorded in transhepatic samples in perfusions of the baboon liver with incompatible blood. (Counts are expressed as a percentage of the original).

	0''	1''	5''	15''	30''	1'	2'	3'	3'05	3¼'	3½'	3¾'	4'
Urea mg/100ml													
(i)	28	24	26	28	31	42	58						
(ii)	30	24	30	26	26	32	42	46	-	48	50	52	58
(iii)	27	25	26	26	26	32							
Ammonia: umols/1													
(i)	,56	,59	,56	,53	,52	,42	,36	-	-	-	-	-	-
(ii)	,26	,22	,22	,18	,26	,21	,20	,16	1,50	1,56	,73	,34	,19
(iii)	,32	,34	,28	,26	,27	,26							
Lactate/pyruvate ratio:													
(i)	18	17	6	7	9	8	7	-	-	-	-	-	-
(ii)	18	18	23	6	3	4	9	4	6	5	7	7	5
(iii)	20	21	20	17	12	12	-	-	-	-	-	-	-

Platelets:

	1''	5''	15''	30''
(i)	73 / 75	75 / 80	67 / 66	70 / 82
(ii)	88 / 77	99 / 72	70 / 78	70 / 60
(iii)	92 / 85	120 / 89	96 / 78	96 / 72

Leucocytes:

(i)	96 / 32	70 / 15	21 / 17	12 / 17
(ii)	90 / 80	88 / 11	26 / 9	14 / 11
(iii)	98 / 75	115 / 119	57 / 24	23 / 16

CHAPTER NINE

DISCUSSION

DISCUSSION:

The objective of this programme of experiments was to find some treatment which would improve the mortality from acute hepatic failure. The use of the isolated perfused pig liver as a hepatic assist still has great appeal which will continue until an "artificial liver" is designed. As the increasing collection of information reveals the complexity of liver metabolism, it is obvious that the chaos resulting from total hepatic destruction cannot at present be controlled with anything but another liver. Thus the rationale for continued effort in the field of hepatic assist is justified, although to date, no series of patients thus treated is sufficiently large to show a significant improvement in survival as a result of the procedure. Indeed, the severity of the disease may be such that after a certain point, there is no treatment which could be effective. Although this point is, at present, impossible to define, earlier institution of hepatic assist, might yet reverse the decline in some patients.

Thus there is a need for a safe, well-functioning system, which could in fact be used earlier without hazard. At present, the pig liver cannot be said to fulfill these criteria, but there are indications that the baboon liver may prove more successful. However the potential virological hazard of the use of these animals must be borne in mind. Several reports exist of human infection from various primates (209), and a recent review has summarised current opinion (246). The hazard of virus infection from African green monkeys was first noted in 1967 when 31 persons from 3 institutions became ill, and 7 died. The incubation period of the illness was 3-9 days, and a maculo-papular rash developed which was non-irritating but associated with fever. There was evidence of involvement of the central nervous and haemopoeitic systems but the greatest effect was noted upon the liver with increase in transaminase yet no jaundice or coma. Death occurred between the 8th and 16th days of illness, as a result of circulatory collapse and coma. Thrombocytopenia was common, and focal encephalitis was noted at autopsy in all patients. In an attempt to prevent transmission of virus from animals to laboratory workers, an isolation period of a minimum of six weeks was advised for any imported animals.

The studies described in this thesis have explored several avenues of investigation of the isolated liver; a few questions have been answered and a great many more posed. These may be broadly grouped as follows:

i) Can further improvement be made in the function of the isolated liver?

ii) Can further contribution be made to solution of the heterograft problem?

iii) How could the use of a satisfactory experimental model of hepatic coma aid further improvements in treatment?

i) **Improvement in liver function:**

Apart from its physiological interest, adequate function of the liver used for hepatic assist is essential. Several types of experiment which may further define the effects of poor preservation of the liver are urgently needed.

(a) A controlled comparison of a variety of liver functions in the intact awake animal and the perfused liver. Studies to date which have explored the metabolism of bromsulphthalein and alcohol (74) and the elimination of galactose (128) have shown a deficit of function in the perfused liver. For greater validity, these studies should be performed with the animal unanaesthetised, and under various conditions of feeding when an even greater deficit might be demonstrated. As an important corollary to the work, attempts should be made to improve the function of the isolated organ whilst such comparative studies are under way. In these instances, studies should be made of changes across the organ as can be performed in the isolated liver, since little information exists in regard to the biochemical effects of the liver alone. An associated investigation during which various periods of inflow occlusion were studied would also provide evidence of the trans-hepatic effects of ischaemia. Most studies to date have been performed using the dog liver (215) and have included splanchnic as well as hepatic ischaemia.

(b) Since, at present, the only measure of assessment of total liver function involves the complexities of transplantation, a study is required of the liver perfused at normal temperature for increasing periods with subsequent re-implantation into a recipient animal. However, it is fully recognised that such procedures are complicated by defects in coagulation (127) which claim the lives of these animals. Hence intensive further study is needed of the coagulation problems resultant upon poor storage of the liver. If the problem is that of failure of synthesis of clotting factors, and endothelial damage resulting from poor preservation, an intensive attempt to improve storage may solve this.

(c) A major effort should be made to improve preparation of the liver. Assessment of the organ just prior to removal might reveal defects which should be rectified before insertion into the perfusion circuit. A period allowed for recovery at that time might improve this "unconventional ischaemia", as might the administration of protective drugs such as hydrocortisone, dibenzyline or chlorpromazine (145). A concerted investigation should be made into the constitution, volume, temperature and form of delivery of the flushing solution, since more adequate protection during short term ischaemia is necessary.

A criticism which might be levelled at the work presented, is that the liver was inadequately washed out on some occasions, and that remaining animal blood might initiate the immune response. This occurred in only a few experiments where the final effluent was

still obviously blood stained, - in the remainder, the liver became the uniform beige and the effluent clear as reported. It was felt that excessive flushing of the liver might be more harmful.

Although Judah has suggested previously and has recently reiterated, that ischaemia commences with perfusion, the addition of various substrates appeared to reverse many of the results of ischaemia (58). In his series of experiments, the addition of αoxoglutarate preserved oxidative phosphorylation; in the current studies, livers to which this substrate was administered in the flushing solution, the perfusate and the infusing medium appeared to function better in those features noted. Certainly the appearance of these organs suggested improvement in perfusion. The use of other substrates or substrate precursors as shown by Krebs (146) may also result in better function.

ii) **The heterograft:**

Heterologous perfusion of the pig liver gave results suggestive of an acute rejection phenomenon. These results were however, not as dramatic as the total cut-off in flow of blood and urine which occurs with heterologous perfusion of the kidney. Nonetheless, the effect upon cellular constituents was similar and had resulted in the initial clinical observation of thrombocytopenia after hepatic assist. The disappearance of platelets and leucocytes paralleled the absorption of antibody and hence could be taken as an index of rejection or its prevention. None of the methods used in an attempt to prevent heterograft rejection resulted in any improvement of the thrombocytopenia, although the use of citrate appeared to prevent the aggregation of leucocytes.

Additional confirmatory evidence of the role of the liver in this response could be obtained with the use of more sophisticated immunological techniques. To date, the presence of platelet thrombi in the hepatic sinusoids has only been inferred but not proven histologically; electron microscopy might reveal these. Also, the use of radio-isotopic labelling of platelets with subsequent autoradiographic exposure of the histological specimens, might demonstrate the site of adherence. Individual labelling of platelets and leucocytes might aid their differentiation.

In these particular studies, a partial solution to the problem of thrombocytopenia has been achieved by choice of livers from a different species. Due to the relative unavailability of baboons especially in other countries, the pig liver would have a place were it not for the acute rejection phenomenon which it provokes. In addition, the heterograft phenomenon remains one of the most vexed in transplantation biology, and its solution would offer a wide range of experiments and therapy. Extra-corporeal organ perfusion with heterologous blood provides a valuable tool for further investigation. At present, the most likely solution appears to be the fractionation of cells or plasma components in an attempt to remove the participants in the response (27), and thereby to prevent the sequelae of the formation of antigen/antibody

complexes. When this technique is perfected beyond its present experimental stage, there is definite clinical application.

(iii) **Acute hepatic failure** - the roles of liver regeneration and an experimental model.

It is certain that a great deal more knowledge is necessary concerning the nature of the biochemical defects resulting from acute hepatic failure, and with further elucidation of these, more rational treatment may be undertaken either with pharmacological means, or with physical removal of toxins or provision of liver function.

At this time, the most likely avenue appears to be that of liver regeneration and its control. Since this is probably the principal reason why most patients do not recover, elucidation of the stimuli and suppressors of liver regeneration may provide a major new means of management.

Meanwhile since a controlled trial of treatment is not possible, and since it would be difficult in any one series to collect sufficient patients to provide a convincing demonstration of the effect of any particular treatment, a satisfactory experimental model acute hepatic failure is required. The use of the rhesus monkey by Trey has provided such a model, but again these animals are generally scarce, and the more available pig should be investigated. Once such a model is obtained, the relative values of the current forms of treatment could be evaluated and the biochemical derangements might be clarified.

In conclusion, therefore, in regard to this study in particular, at present the pig liver cannot safely be offered by this laboratory for the treatment of acute liver failure. Some solution seems to lie in the use of the baboon liver as regards the prevention of thrombocytopenia, but the biochemical function of these livers remains to be demonstrated in clinical application.

Appendix of methods:

biochemical

haematological

immunological

Biochemical methods:

1. Astrup determinations were made on a Radiometer apparatus with derivation of the partial pressure of carbon dioxide, the bicarbonate and the base excess from samples equilibrated with known concentrations of carbon dioxide (4% and 8%) using the Siggaard Andersen nomogram. The partial pressure of oxygen was measured directly with a Clarke electrode.

2. Oxygen consumption was calculated according to the method of Selkurt and Brecher, but several values for pig blood were assumed to be similar to those accepted for human blood - viz. the oxygen carrying capacity of haemoglobin - 1,34ml oxygen/g haemoglobin, and oxygen solubility in plasma 0,003ml/mm pO_2. Haemoglobin was measured according to the method of Sheard and Sanford using a Leitz photometer, and oxygen saturation was determined using a Radiometer oximeter. The final calculation was as follows:

   Oxygen carried by haemoglobin:-
   (Portal vein O_2% saturation x flow/min + Hepatic artery O_2% saturation x flow/min minus Inferior vena cava O_2% saturation x flow/min) x haemoglobin g/100ml x 1,34, PLUS

   Oxygen dissolved in plasma:-

   (Portal vein pO_2 x flow/min + Hepatic artery pO_2 x flow/min - Inferior vena cava pO_2 x flow/min) x 0,003

   The final result was expressed as millilitres of oxygen consumed per kilogram liver wet weight.

3. Sodium and potassium in plasma and bile were measured by flame photometry with lithium as internal standard, using a Baird Atomic model KY 1 flame photometer. Chloride was determined coulombmetrically on an Aminco Cotlove automatic titrator after the design of Cotlove (Cotlove and Nishi).

4. Glucose was measured by the alkaline ferricyanide AutoAnalyser method (Hoffman).

5. Osmolality of plasma and bile was calculated from the depression of freezing point using an Advanced Instruments or Fiske osmometer.

6. Lactate determinations were made initially using the method of Blair and Summerson as modified by Huckabee (Huckabee) and pyruvate by the enzymatic method of Segal, Blair and Wyngaarten. Just prior to the experiments described in phase IV, the improved methods of Bucher were employed and continued for the remainder of the study.

7. Total protein and albumin were measured by the biuret method before and after precipitation with sodium sulphite, and the globulin was obtained by difference (Wolfson).

8. Alkaline phosphatase determinations used phenolphthalein monophosphate as the substrate (Babson) the method being modified for use on a Technicon AutoAnalyser. Results were expressed as Shinowara-Jones-Reinhart units.

9. Aspartate transaminase was determined according to the method of Karmen.

10. Cholesterol measurement employed the Liebermann-Burchard reaction (Pearson).

11. Bilirubin in plasma and bile was measured by methods of Malloy and Evelyn - using the diazo reaction for plasma levels and direct oxidation with hydrogen peroxide for bile.

12. Urea was measured on an AutoAnalyser using urease and the Berthelot reaction as described by Chaney and Marbach.

13. Plasma haemoglobin was determined spectrophotometrically (Hunter, Grove-Rasmussen and Soutter).

14. Plasma ammonia was measured using a combination of the methods of Kaplan, Chaney and Marbach. Under anaerobic conditions, blood samples were added to equal volumes of 15% trichloro-acetic acid for protein precipitation. After centrifugation, 0,5ml supernatant was mixed with 0,5ml phenol/sodium nitroprusside reagent, and 1,0ml alkaline hypochlorite. (The commercial bleach, Nomisol, was found to contain the correct concentration of hypochlorite). After a ten minute incubation period in a water-bath at 37°C, the absorbance was read spectrophotometrically at 625μ. Final calculation was made from a standard curve.

Haematological methods:

15. Platelet and leucocyte counts were made visually using an improved Neubauer counting chamber. The diluent for platelet counting was 1% Ammonium oxalate. Although the reported error of this form of counting is 30% it was felt that this was justified when compared with the inaccuracy involved in the use of mechanical counting where error could arise from particles of the counted size not being platelets but rather fragments of cells resulting from trauma. In addition, clumping of cells could be detected visually. In an attempt to reduce the error in counting, all samples were counted in duplicate and the mean recorded.

16. Complement was determined according to the method of Kabat and Mayers using haemolysis of sensitized sheep erythrocytes. The pH of the diluent buffer used in determination of pig complement was changed from 7,5 as used for human complement estimations to 7,3 to obtain optimal values.

Immunological methods:

17. Antibody testing.

(a) Preparation of lymphocytes (Kissmeyer-Nielsen and Kjerbye) - from 20ml defibrinated blood, 8ml was added to pre-warmed iron powder and 3ml methyl cellulose. This mixture was rotated at 37°C for 30 minutes to allow phagocytosis of iron by erythrocytes. Subsequent sedimentation for 30 minutes resulted in a supernatant fluid free from erythrocytes (Coulson and Chalmers). Two millilitres of this supernatant was then layered onto 1,5ml Ficoll Isopaque (Boyum). After centrifugation at 15 000 rpm for 20 minutes, an isolated layer of lymphocytes could be removed and was washed twice with Hanks balanced salt solution. Centrifugation between these two washes was conducted at 1 200 rpm for 8 minutes. After draining the Hanks, the cells were resuspended in 2 drops of Hanks which resulted in a dilution of 12 000 lymphocytes/mm_3. These cells were stored on ice at 4°C until use. For the final assessment of lymphocytotoxicity of plasma, 3 drops absorbed rabbit complement (see below) were added to the suspension of cells. One microlitre of decomplemented test serum was dispensed into Moller-Coates trays and one microlitre of the lymphocyte/complement mixture was added. After incubation at 37°C for 30 minutes, 0,5ml. 1% Trypan blue was added to each well and the tray re-incubated at 20°C for 30 minutes. Cytotoxicity was determined using an inverted vision microscope, and was scored as follows:- Negative = less than ten per cent cells which were not capable of excluding the dye; + = 10-20%; ++ = 21-50%; +++ = 51-74%; ++++ = 75-100%.

(b) Preparation of leucocytes - from 20ml defibrinated blood, 8ml was added to 3ml methyl cellulose and allowed to sediment for 30 minutes at 37°C. One half millilitre was removed from the uppermost layer and was dispensed in 2 microlitre aliquots into Terasaki trays. Two microlitres of decomplemented test serum was added and the trays were incubated for 105 minutes at 37°C. After addition of one microlitre of Toluidine blue and further incubation, aggregation was noted and scored in a similar manner to that described above. *Test sera* were prepared by centrifugation of plasma at 4 000 rpm for 2 minutes, decomplementation by incubation at 56°C for 30 minutes and further centrifugation in a microcentrifuge for 5 minutes.

Preparation of absorbed rabbit complement:

Five millilitres of blood from the experimental animal was collected with 0,5ml Heparin without phenol preservative (Thromboloquine - Organon - 50mg/ml). After centrifugation at 2 000 rpm for 10 minutes, the buffy coat was removed and added to an equal volume of rabbit complement. The mixture was allowed to stand for 120 minutes at 4°C and centrifuged on ice for 10 minutes at 3 000 rpm. The supernatant was used as described above, or stored at 4°C.

Heterophile antibodies:

Heterophile antibodies were detected in plasma samples by the method of Rapaport et al. Sheep cells were obtained from Karl Bremer Hospital, rat, guinea pig and baboon cells from the Animal House, University of Cape Town and human cells were prepared from pooled group O human blood. All cells were washed five times with phosphate-buffered saline, pH 7,2, and were diluted to 1%. Serial dilutions of test plasma samples were made in phosphate buffered saline. For the definite test, 0,3ml cell suspension was mixed with 0,3ml plasma dilution, incubated for 2 hours at 22°C, and was then centrifuged and observed microscopically for evidence of erythrocyte aggregation.

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Summary of a Thesis to be presented to the University of Cape Town
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by Rosemary Hickman.

PIG LIVER PERFUSION - A Role in Hepatic Assist ?

The pig liver has been used in many institutions as a means of treatment of acute liver failure. Since the incidence of this complication is very high at Groote Schuur Hospital, and because of the associated mortality of 80 to 90%, the model of pig liver perfusion was developed in the laboratory in 1969.

After physiological studies using human blood in comparison with initial perfusion studies which had used pig blood, the procedure was applied as hepatic assist to 4 patients in fulminant hepatic failure. Although none survived, there was sufficient improvement in their level of consciousness to encourage the technique to be pursued. However, all clinical applications had been complicated by the development of thrombocytopenia. This problem had not been reported from other laboratories and hence the clinical programme was terminated while the platelet decline was investigated.

Two possible explanations existed. The incorporation of a bubble oxygenator in the circuit was excluded as a cause by experiments using blood in the circuit alone without a liver.

It became evident from estimation of platelets in blood samples taken across the liver, that a significantly greater fall occurred when the pig liver was perfused with human blood as compared with pig blood. This observation was interpreted as an acute rejection phenomenon, more especially as an associated decrease in levels of complement and antibodies was noted.

Attempts were made to prevent this acute response, using pharmacological means but none was successful. An alternative was to use livers from different animals. In comparison with the pig liver, no difference was noted with the use of the calf or lamb liver. The baboon liver showed a significant improvement in the fall in platelets. In addition,

no significant decrease occurred in complement levels and no antibodies were detected in human blood against baboon tissue.

In conclusion, it has been decided as a result of these experiments, that the baboon liver should be used for hepatic assist when practised in this Unit. In order to diminish the hazard of viral spread from animals to patient or personnel, baboons to be used to donate livers, will have been kept in isolation for a period of at least six months.