ISOLATED PERFUSED LIVER TECHNOLOGY FOR STUDYING METABOLIC AND TOXICOLOGICAL PROBLEMS

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SUMMARY

The isolated perfused liver system is a versatile model for investigating the effects and mechanisms of action of hepatotoxins and the metabolism of endogenous and exogenous compounds. The interpretation of metabolic data and apparent toxic events is dependent upon the viability and reproducibility of the model. In this study, a new approach has been undertaken to assess the viability of isolated liver preparations. This has involved the continuous monitoring of multistage processes namely, the synthesis and secretion of radiolabelled proteins, glycoproteins and lipoproteins on the one hand and the uptake of macromolecules by receptor-mediated endocytosis on the other.

The consistency of these complex integrated processes from one liver to another and in particular the steady-state rate of production of radiolabelled macromolecules over 6h perfusion periods suggests that this model can be used with confidence for metabolic/toxicological investigations. The selectivity of the responses to chemical challenge(s) shows that this system can be exploited for (a) screening potential hepatotoxins; (b) identifying areas of metabolism which are affected and (c) advancing basic knowledge of liver biochemistry.

I. INTRODUCTION

Organ perfusion techniques bridge the methodological gap between whole animal models and isolated cell preparations. Their advantages and disadvantages have been discussed extensively over the years and clearly the relative merits and demerits of usage depend on the nature of the problem under investigation. The major conceptual dilemma arises from the assertion by advocates of perfusion techniques that many processes are not amenable to accurate measurement and manipulation in the intact animal because of the competition and integration with other tissues and organs. Ironically, the same logic in reverse is used by sceptics on the grounds that if the rates of certain organ-related processes cannot be accurately recorded in the whole animal, it is impossible to be certain that the perfused organ is operating within normal limits. In short, the viability and normal functioning of the perfused organ is often in doubt. Nevertheless, the demand for advances in perfusion technology is increasing for a number

of reasons not the least of which is the knowledge that is required for *ex vivo* preservation for the successful prosecution of organ transplantation programmes. In addition, biochemists are turning increasingly to more complex systems to study integration kinetics in order to appreciate the whole metabolic fabric.

There is no doubt that the use of isolated organs to provide information about the biochemistry of complex systems has been immeasurably advanced by the development of increasingly complex technology. This is particularly well illustrated by the development of the isolated perfused liver system as an investigative technique. The liver has proved to be a particularly amenable organ for *ex vivo* investigation but as with any isolated organ it has been important to ascertain that normal functions, are demonstratable and furthermore that they can be interpreted in terms of whole animal biochemistry and physiology bearing in mind the absence of neural and hormonal influences. Some of the major advantages of the system for investigating hepatic function are that the various liver cells are present in normal polarity, the physiological relationships are maintained and cellular kinetics and cellular inter-dependence can be preserved.

Perfusion of the liver through its vascular bed was reported by Claude Bernard as early as 1895 and since then the value of the method as a means of investigating specific problems has been amply demonstrated by numerous research groups. An impressive range of normal hepatic functions has been explored and the benefits that have accrued in understanding normal liver physiology and biochemistry are legion (Table 1). In particular Thurman and coworkers /28/ have described how chemical toxins can be used as selective inhibitors in the perfused liver system to unravel the mechanisms of hepatic biochemical functions. In addition, the system has been usefully employed in studying various abnormal and disease states, for example, diabetes /29,30/, hyperbilirubinaemia /31/, tumour /32,33/, uraemia /34,35/ hypoxia /36/, cirrhosis /37/, and in studies on regeneration of liver /38,39,40,41/. The majority of research has been carried out using rat liver but many other mammalian species have been used, for example, pig /42/, cow /43/, guinea pig /44/, dog, /45/, mouse /46/, rabbit /47/.

The isolated perfused liver system represents a versatile model for investigating the effects of toxins but quite clearly it has been essential to establish the viability and reproducibility of the model before the validity of such investigations can be established. Historically, many

TABLE 1

Hepatic functions studied using the isolated perfused liver

	Selected References
Biosyntheses	
Plasma protein synthesis	1, 2, 3, 4.
Gluconeogenesis	5 6, 7, 8
Glycogen synthesis and degradation	9, 10, 11, 12, 13, 14.
Fatty acid synthesis lipid, metabolism and ketogenesis	15, 16, 17, 18, 19, 20.
Biotransformation	
Mixed-function oxidation	21, 22, 23
Conjugation	24, 25, 26.
Macromolecular degradation and reutilisation	27.

studies have utilised various indices to assess the viability of the preparations (Table 2).

Parallel with the development of the isolated liver perfusion technique, the range of applications has been extended particularly into areas of toxicology. In broad terms the technique has been used for (a) investigating the effects of toxins on normal liver functions and cell viability and (b) investigating the range, capacity and rates of metabolism of exogenous compounds and the partitioning of metabolites between bile and perfusate. These two aspects invariably overlap when the objective is to describe the mechanism of action of a particular hepatotoxin.

The central dilemmas in identifying and monitoring hepatotoxins are the choice of a model system and the choice of the parameters that can be measured and used diagnostically. Diagnostic indicators of liver damage in plasma, such as bilirubin, alkaline phosphatase, gamma-globulin, albumin, γ-glutamyl transferase, aspartate aminotransferase, alanine aminotransferase are well recognised and are particularly important in the clinical assessment of liver function. However, these indicators have limited usefulness in the present context because they signify severe and gross cell damage and provide little information about the early biochemical events associated with toxicity.

TABLE 2.

Indices used to assess the viability of isolated perfused liver preparations

1.	Physiological Indices	Selected References
	(i) Pressure	48, 49, 51;
	(ii) pH	50.
	(iii) PO_2	51, 52, 53.
	(iv) PCO_2	53.
2.	Chemical Indices	
	Release of enzymes	
	Aspartate aminotransferase,	
	Alanine aminotransferase,	
	Lactate dehydrogenase,	54.
	5'Nucleotidase	
3.	Histological Examination of Ultra Structure	
	Light and electron microscopy	55.
4.	Endogenous Biochemical Indices	
	(i) Lactate/pyruvate ratio	52.
	(ii) ATP/ADP ratio	5.
	(iii) Glucose production	48.
	(iv) Urea production	48, 5.
	(v) NAD^+/NADH: $NADP^+$/NADPH ratio	56.
5.	Exogenous Markers	
	(i) Bromosulphophthalein uptake	57.
	(ii) Galactose uptake	58.

The strategy in these laboratories has been not only to extend the range of viability tests but to develop a new approach. This has involved the monitoring of integrated hepatic biochemistry. Potential hepatotoxins have been monitored routinely using four basic hepatic functions:

1. Protein synthesis and secretion
2. Glycoprotein synthesis and secretion
3. Lipoprotein synthesis and secretion
4. Receptor-mediated endocytosis

Protein, glycoprotein and lipoprotein synthesis and secretion were monitored by measuring the rate of incorporation of radio-labelled

precursors; ^{14}C-leucine, ^{3}H-*N*-acetyl glucosamine or galactosamine and ^{14}C-acetate or ^{14}C-mevalonate respectively /59,60,61/. Receptor-mediated endocytosis /62/ was monitored by measuring the rate of uptake of horse radish peroxidase or ^{125}I-labelled asialoglycoproteins. The rationale for the selection of these is that the four processes embrace a significant proportion of hepatic metabolism, each requiring the inter-dependent operation of a number of multi-stage metabolic pathways and membrane activities. If the primary aim is to pinpoint the site(s) and mechanism(s) of action of a particular drug, industrial chemical, food additive, herbicide or pesticide, then the responses of these four processes to a potential toxin are invaluable because the results will either eliminate large areas of hepatic metabolism from any further consideration or the results will show how sensitive these multi-stage processes are to any chemical challenge.

The purpose of this review is two-fold. Firstly, it investigates the viability and reproducibility of isolated perfused liver preparations and secondly, it evaluates the use of the model system for (a) advancing knowledge of liver physiology and biochemistry and (b) assessing its potential for identifying hepatoxins and their modes of action.

II. EXPERIMENTAL PROCEDURES

2.1 Perfusion System

The essential features of the perfusion apparatus used in this Department are shown in Figure 1.

2.2 Perfusates

The perfusate most commonly used in this laboratory is based on bovine red blood cells suspended in supplemented Krebs-Ringer bicarbonate buffer /50/. Heparinised whole rat blood is also used occasionally and although there are differences in the rates of metabolism of some compounds (unpublished data) using whole rat blood compared to bovine cells, the artificial medium described in this review has proved satisfactory for studying protein, glycoprotein and lipoprotein production and receptor-mediated endocytosis by the isolated perfused rat liver /63/.

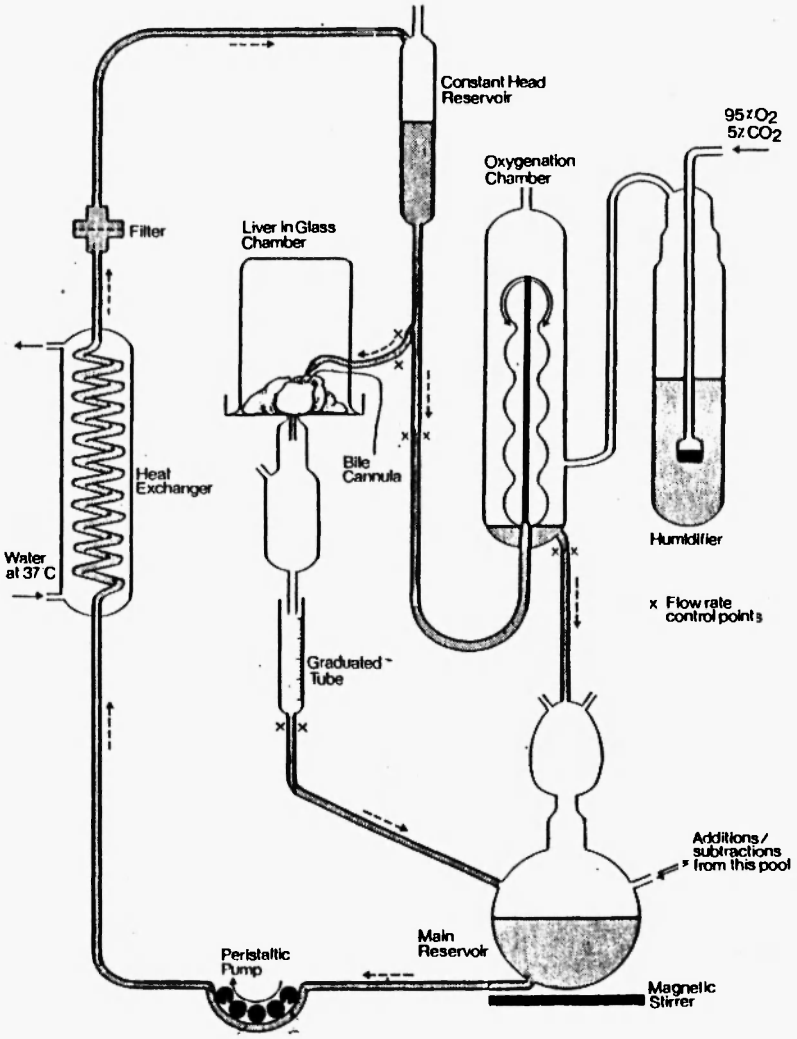

Fig. 1: Diagrammatic representation of the perfusion apparatus.

i. Separation of red blood cells from bovine blood

Blood from cattle is collected at slaughter into containers primed with heparin (6,000 NIH Units/litre blood). The blood is transported immediately to the laboratory where it is filtered through several layers of surgical gauze to remove any clots and then centrifuged (1500g) for 20 min at 5°C. The plasma and buffy coat are removed as completely as possible by aspiration; the remaining cells are resuspended in an equal volume of Ringer solution (NaCl 9g; KCl 0.3g; $CaCl_2$ 0.25g; $NaHCO_2$ 0.2g per litre, pH 7.4) containing heparin (100 NIH Units/ml) and centrifuged (1500g) for 20 min at 5°C. The washing procedure with Ringer solution (but without added heparin) followed by centrifuging and removal of supernatants together with any remaining buffy coat is repeated twice.

To each 100ml portion of packed red blood cells is added 2ml of a solution containing glucose (95mg) streptomycin sulphate (7.5mg) and penicillin G(42μg). The cells are stored at 4°C until required and used within ten days of preparation.

ii. Preparation of the perfusate

The packed red blood cells (100ml) are resuspended in an equal volume of Ringer solution and centrifuged (1500g) for 20 min at 5°C, the supernatant is aspirated and the whole procedure repeated. Finally the red blood cells are suspended in Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 3% (w/v) bovine serum albumin so that the final packed cell volume is 35%. Glucose (250mg), heparin (625 Units), penicillin G (3,340 Units) and insulin (8 Units) are added to 150ml of perfusate. The isotonic saline circulating at 37°C in the perfusion apparatus is removed and the perfusate is introduced into the apparatus and circulated prior to commencement of surgery. This allows the perfusate to be fully oxygenated and equilibrated at 37°C before attaching the liver.

2.3 **Surgical Procedures**

The surgical procedure for isolating the liver is essentially that described by Miller /50/. The rat liver donor is anaesthetised with either ether or phenobarbitone (50mg/Kg body weight), and the peritoneal

cavity is opened by an incision along the bloodless mid-abdominal line. Lateral cuts are made in the abdominal wall just below the thorax and the walls retracted with haemostats. The common bile duct is cannulated with polyethylene tubing (int.dia. 0.28mm, ext.dia. 0.61mm; Portex Ltd., Hythe, Kent, U.K.) towards the liver. The pancreatico-duodenal artery and vein are ligated close to the portal vein and two loose ligatures are placed around the hepatic portal vein. The inferior vena cava is located and a ligature is placed around it. Heparin (500 Units/0.1ml saline) is administered intravenously.

The hepatic portal vein and hepatic artery are ligated and the portal vein cannulated towards the liver with a cannula (int.dia. 1.67 mm, ext.dia. 2.42mm) attached to a 50ml syringe containing Krebs-Ringer bicarbonate buffer at 37°C. The liver is perfused slowly with buffer via the portal vein cannula during subsequent surgical procedures to remove rat blood from the liver. The inferior vena cava is ligated and divided between the liver and kidney.

The thorax is opened and perfusion of the liver via the hepatic portal vein is discontinued. The superior vena cava is cannulated towards the liver with polyethylene tubing (int.dia. 1.67mm, ext.dia. 2.42mm), its connection with the heart is severed and perfusion of the liver with Krebs-Ringer bicarbonate solution is resumed. These procedures in the rat are also used for guinea-pig and rabbit except that the gall bladder is ligated prior to cannulation of the bile duct.

The liver is rapidly dissected from the body cavity, placed on the supporting tray and perfused with the remainder of the Krebs-Ringer solution. The organ is then transferred to the perfusion apparatus and receives oxygenated perfusate via the hepatic portal vein cannula. The position of the liver is adjusted so that a steady flow of perfusate through the liver is achieved (25-50ml/min). A constant head of pressure (12-14cm of water) is maintained throughout the perfusion.

The physiological status of the liver is assessed during perfusion by monitoring bile flow (every 10 minutes), perfusate flow through the liver (every 30 minutes), oxygen consumption, pH of the perfusate and by the general appearance of the organ (uniform colouration and absence of oedema). In addition, the haemolysis of red blood cells is monitored throughout perfusion and the release of liver cytoplasmic enzymes into the perfusate has also been measured.

2.4 Continuous Infusions

When monitoring protein, glycoprotein or lipoprotein production by the isolated perfused liver, a solution containing glucose (250mg), penicillin (3,340 Units), insulin (8 Units) and heparin (625 Units) in Krebs-Ringer bicarbonate buffer (10.5ml) is infused continuously into the main reservoir at a rate of 1.5ml/h during the perfusion. Solutions of essential and non-essential amino acid mixtures (Table 3) are also added to the perfusate throughout the perfusion period, either as a continuous infusion (approximately 1 ml/h) in the case of lipoprotein production or as successive pulse doses in experiments that monitor protein and glycoprotein production (5ml initially and then 0.5ml/30min). The radiolabelled precursors for protein, glycoprotein and lipoprotein synthesis are added to the mixture of essential amino acids.

TABLE 3

Composition of amino acid mixtures used in perfusion experiment

Essential amino acids	mM	Non-essential amino acids	mM
L-arginine HCl	13.2	L-glutamic acid	69.3
L-isoleucine	20.7	L-hydroxyproline	9.8
L-methionine	25.5	L-serine	5.8
L-valine	18.0	L-tyrosine	15.0
L-histidine-HCl	6.6	L-proline	27.8
L-leucine	4.6	L-alanine	6.7
L-lysine-HCl	23.1	L-cysteine-HCl	7.1
L-phenylalanine	18.2	L-aspartic acid	9.8
L-tryptophan	3.1	L-glycine	46.5
L-threonine	25.5		

Mixtures of essential and non-essential amino acids are suspended separately in water (10ml) and dissolved by adding a minimal volume of 2M-NaOH with warming. Ringer solution is added to achieve the required volume. The pH of the essential amino acid solution is adjusted to 7.4 and the non-essential amino acid solution to 9.2 with 2M-HCl. The solutions are stored at -20°C until required when they are warmed to 37°C and the pH of the non-essential amino acid solution is adjusted to 7.8 with 2M-HCl immediately prior to use.

III. PROTEIN AND GLYCOPROTEIN PRODUCTION BY THE ISOLATED PERFUSED RAT LIVER

After 10-15 min perfusion, when it is established that perfusate flow is greater than 25ml/min and that bile flow is continuous, pulse doses of both non-essential and essential amino acids containing either ^{14}C-leucine (4uCi) or ^{3}H-*N*-acetyl glucosamine (40uCi) are added to the perfusate. Further additions of the amino acids and radiolabel (0.5uCi of ^{14}C-leucine or 5uCi of ^{3}H-*N*-acetyl glucosamine) are added every 30min for the duration of the perfusion (6h). Aliquots (usually 1ml) of the perfusate are removed every 30min, centrifuged (1500g), and the supernatant (plasma) assayed for protein-bound radioactivity. The incorporation of radiolabel is usually expressed as dpm/mg dry wt. protein/g liver. After an initial lag period of approximately 30-60min, a steady state rate of incorporation of isotope is established (up to 6h). By adding potential hepatotoxins after steady state has been attained (i.e. 1.5-3.0h after the onset of perfusion) each liver acts as its own control. Figures 2 and 3 show typical progress profiles for the incorporation of ^{14}C-leucine and ^{3}H-*N*-acetyl glucosamine in control livers and in the presence of ricin, a potent inhibitor of protein synthesis.

IV. LIVER PERFUSION VERSUS HEPATOCYTES IN PROTEIN AND GLYCOPROTEIN PRODUCTION AND RESPONSE TO POTENTIAL TOXINS

For this comparative work, hepatocytes were isolated essentially as described by Berry and Friend /64/. Protein and glycoprotein production were measured by monitoring the incorporation of radiolabel from either ^{14}C-leucine or ^{3}H-*N*-acetyl glucosamine as already described for the isolated perfused liver system.

In contrast to the isolated perfused liver preparations in which the levels of protein and glycoprotein production were remarkably constant from one liver to another ($1.5\text{-}1.6 \times 10^{-5}$, μCi/mg protein/g wet liver wt after 6h), isolated hepatocyte preparations showed considerable variation (up to 14 fold) at 4h. In addition the response to ricin was also variable with some preparations of hepatocytes being markedly less sensitive to ricin even at relatively high concentrations. The sensitivity to ricin

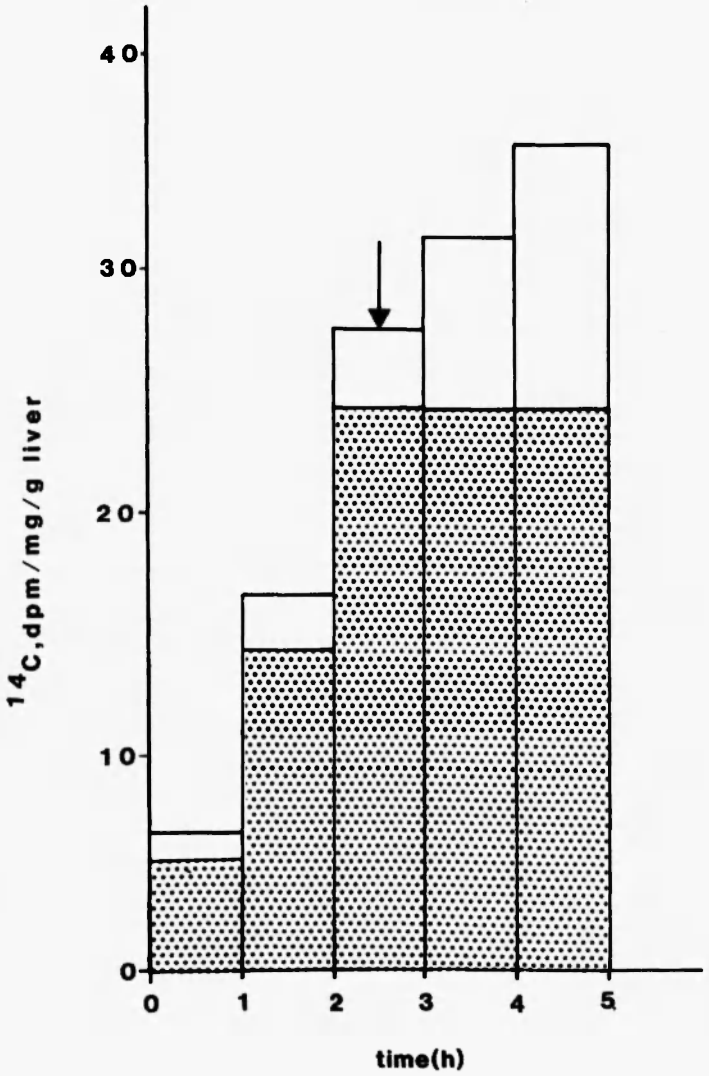

Fig. 2: The incorporation of ^{14}C into secreted proteins produced by isolated perfused rat livers supplied with ^{14}C-leucine. The figure shows two typical perfusion experiments, one in which ricin was not added (□) and the other (■) in which ricin (0.5nM) was added (↓) to the perfusate at 2.5h after the onset of perfusion. In this way each ricin treated liver has its own control period of incorporation prior to the addition of toxin.

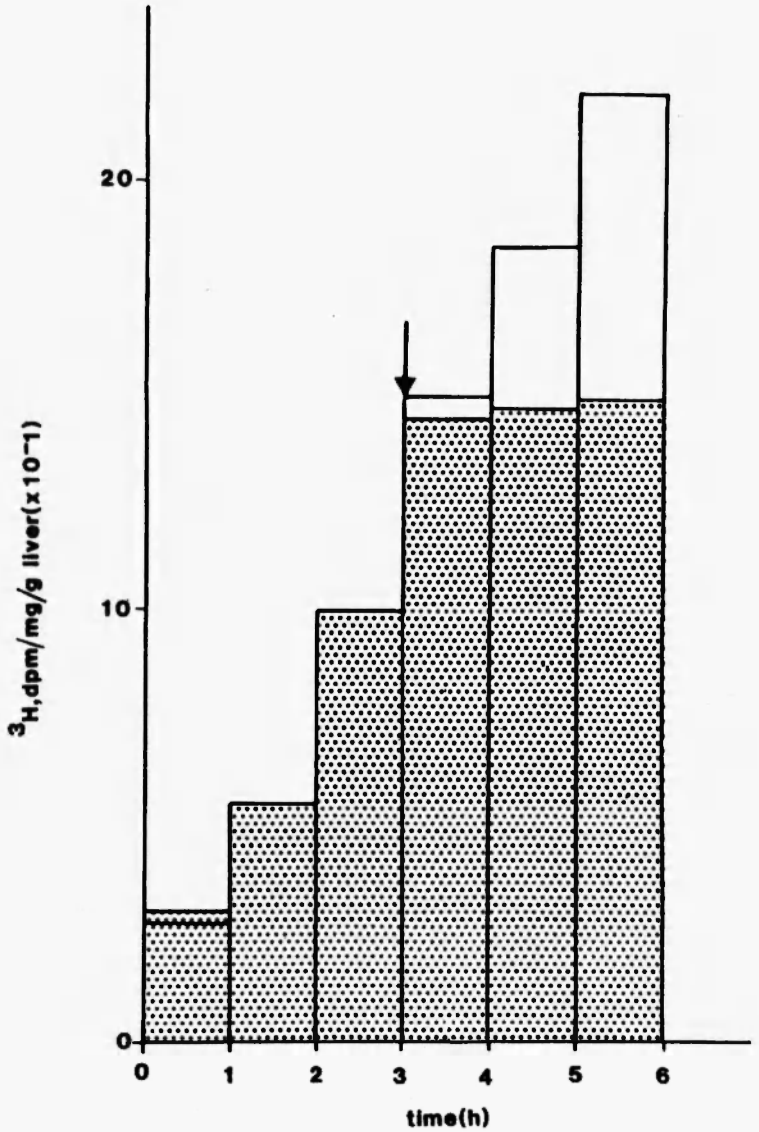

Fig. 3: The incorporation of ^{3}H into secreted glycoproteins produced by isolated perfused rat livers supplied with N-acetyl [^{3}H] glucosamine. The figure shows two typical experiments one in which ricin was not added (□) and the other (■) in which ricin (0.5nM) was added (↓) to the perfusate at 3h after the onset of perfusion.

appears to be dependent upon the collagenase preparation used in the cell isolation procedure. For example, in our hands, cells prepared using some collagenase preparations showed very little inhibition (20%) of ^{14}C-leucine incorporation into protein even in the presence of 10uM ricin (1000 x more concentrated than in liver perfusion experiments), whereas hepatocytes isolated using other collagenase preparations showed up to 80% inhibition at the same concentration of ricin (Fig 4.) Presumably the variation in results between different hepatocyte preparations and the variation between hepatocytes and the isolated perfused liver are due to changes in cell surface properties caused by the isolation procedures.

A further crucial consideration emerges with respect to the wide variations in the levels of protein production by different hepatocyte preparations. Cell preparations, judged to be viable by dye exclusion, frequently produce very little protein. For example hepatocyte preparations with an initial viability value of 90% when assessed by trypan blue exclusion, and maintained to within 10% of the initial value over 4h showed no incorporation of ^{14}C-leucine into protein over 4h. It would appear that measurement of protein production is a more sensitive and reliable indicator of cell viability than the widely used dye exclusion test. Furthermore, our studies have shown that compromised cells, identified by these low levels of protein production are more susceptible to a toxic challenge.

Other principles are highlighted by comparing data obtained from the isolated liver perfusion and isolated hepatocytes in the presence of the anti-oxidant, butylated hydroxytoluene (BHT) and the anti-depressent, imipramine. Figure 5 compares the effect of BHT on protein production in the isolated perfused rat liver with that in isolated hepatocytes.

Whereas protein synthesis is inhibited, approximately 80%, by 0.6mM BHT in hepatocytes, the same concentration has no effect on the whole liver. Similar observations were made with imipramine, which at 0.6mM inhibited protein production by hepatocytes by 50% but multiple doses of 0.1mM imipramine had no effect on total protein or glycoprotein production in the perfused liver. The different responses of the two systems to BHT and imipramine might be due to the inability of hepatocytes to remove drug and/or its metabolites from the immediate environment. In the perfused liver system, the structural integrity of the organ ensures that biliary secretion is unimpaired so

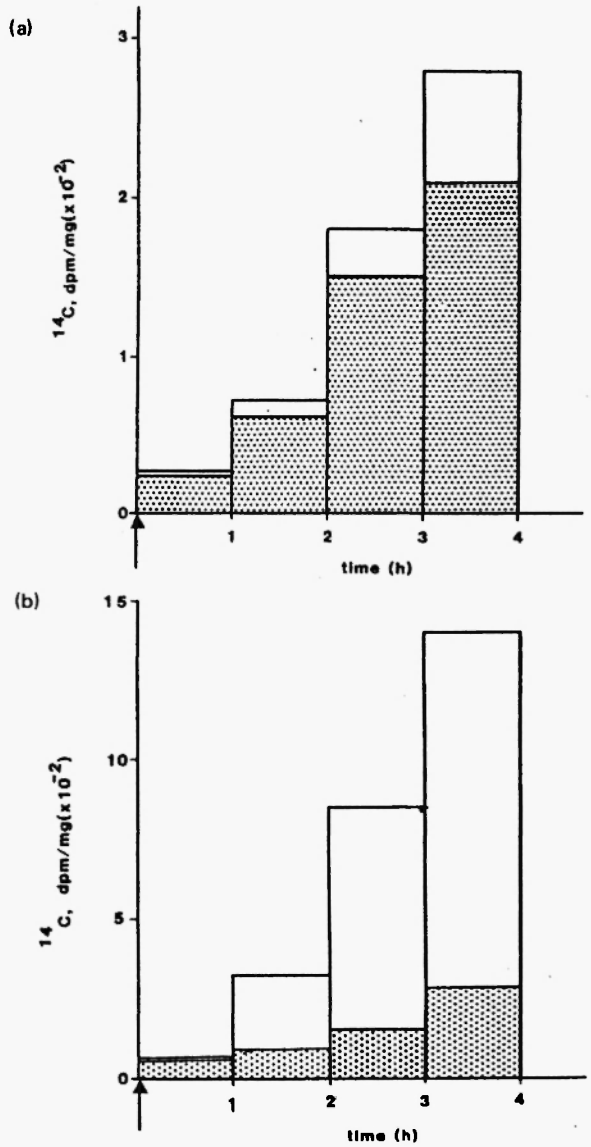

Fig. 4: The incorporation of ^{14}C into secreted protein produced by isolated hepatocytes incubated in the presence of ^{14}C-leucine (□, control cells; ■ test cells in the presence of 0.8uM ricin added at zero time as indicated by ↓). The hepatocytes were prepared (a) using collagenase type 1 preparation obtained from Sigma Chemical Co. Ltd., U.K. and (b) using a collagenase preparation obtained from Boehringer, GmBH, W. Germany.

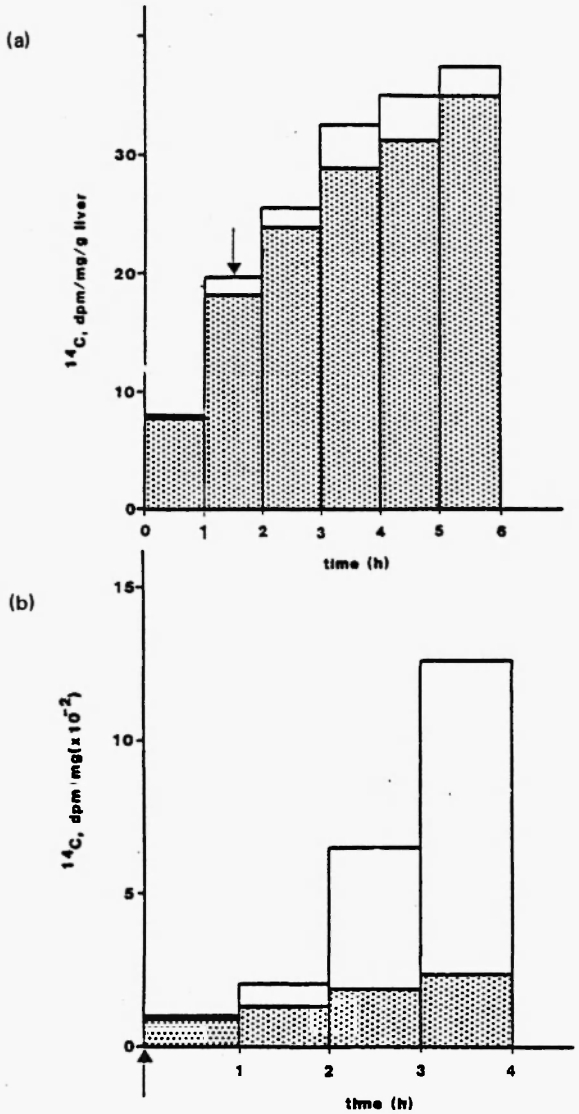

Fig. 5: The effect of BHT on the incorporation of ^{14}C from ^{14}C-leucine into secreted protein produced by (a) the isolated perfused liver (□, control liver; ■, test liver in the presence of BHT (0.6mM) added at 1.5h as indicated by ↓ and (b) isolated hepatocytes (□, control cells; ■, test cells incubated in the presence of BHT (0.6mM) added at zero time as indicated by ↑).
All experiments were carried out in duplicate.

that the blood level of drug equivalents drops rapidly as it would *in vivo*. In hepatocyte preparations, drug metabolites are released into the medium and because the polarity of the cells is lost, both canalicular and sinusoidal surfaces are exposed to parent compound and metabolites. Clearly, the isolated organ and the isolated cell systems are quite different. The advantages of the more economical hepatocyte system for data collection has obvious attractions. However, economy is of no significance if hepatocytes yield false positive results and it is only when it has been established that the whole organ and isolated cells respond to the same dose of toxic challenge that hepatocytes can be used with any degree of confidence.

V. LIPID PRODUCTION BY THE ISOLATED PERFUSED RAT LIVER

Livers are perfused with 150ml of perfusate containing essential and non-essential amino acids, glucose, heparin and insulin. In addition, a mixture of insulin approximately (0.23units/ml) and ^{14}C-acetate (0.5uCi, umoles/ml) in Krebs-Ringer buffer is added as a continuous infusion (1.5ml/h) into the perfusate throughout the perfusion. Aliquots (3ml) of perfusate are collected every 30min and the cells separated by centrifuging. Aliquots (1ml) of the plasma are extracted with chloroform and the lipids subjected to tlc together with authentic markers for free fatty acids, triglycerides, free and esterified cholesterol and phospholipids. After spraying the areas containing the authentic markers with anilino-1-naphthalene sulphonic acid (0.2% in methanol) the appropriate test areas are removed from the tlc plates. The radioactivity associated with each lipid fraction is expressed as a percentage of the total ^{14}C-labelled lipids. In control experiments the rate of incorporation of ^{14}C-acetate into total lipid and triglycerides was approximately linear between 1-5h perfusion. However the rates of incorporation varied from one perfusion to another by as much as two fold. After 5-6h perfusion the majority of the radioactivity (approx. 75% of total lipid) was associated with the triglycerides; 12% with free fatty acids, 6% with phospholipids and 2% with free and esterified cholesterols. Figure 6 shows the incorporation of ^{14}C-acetate into plasma triglycerides in control livers and test livers to which the anti-hyperlipidaemic drug gemfibrozil was added to the perfusate 2.5h

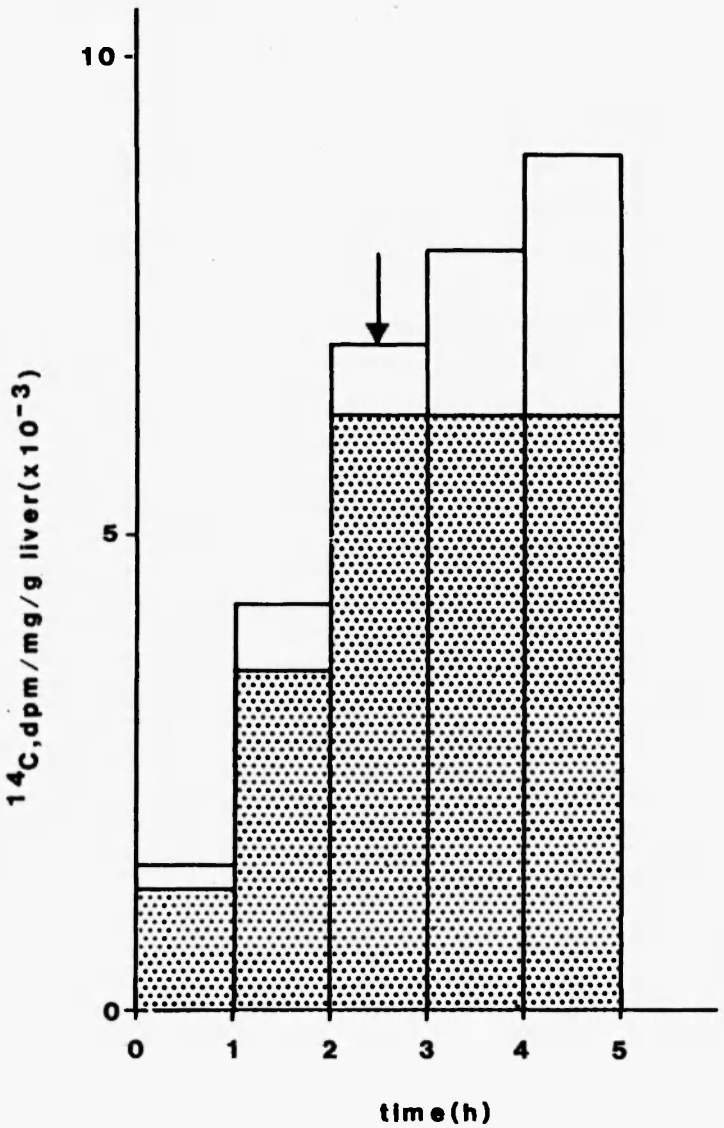

Fig. 6: The effect of gemfibrozil on the incorporation of ^{14}C from ^{14}C-acetate into triglycerides by the isolated perfused liver. (□, control liver; ■, test liver in the presence of gemfibrozil, 1.4mM, added after 2.5h as indicated ↓).

after the onset of perfusion. Under these conditions, the production of ^{14}C-labelled triglycerides was greatly inhibited by gemfibrozil, demonstrating that the isolated system mimics the effect of the drug *in vivo* and importantly, pin-points its mode of action.

The merits of the isolated perfused liver system for identifying and monitoring acute effects are fully substantiated by this example However, other studies with gemfibrozil have shown that the effects of longer term dosing in the whole animal can also be investigated using the system. Thus, in additional experiments, animals were dosed daily for 14-17 days with gemfibrozil and the livers were perfused in the presence of ^{14}C-acetate to monitor lipid production. With this protocol also, it was shown that the incorporation of ^{14}C-acetate into plasma triglycerides was reduced. These findings also illustrate how short-term toxicity testing might be relevant to the pharmacological action of the drug in the whole animal.

VI. RECEPTOR-MEDIATED ENDOCYTOSIS IN THE PERFUSED RAT LIVER

Absorptive pinocytosis may be subdivided into endocytosis of markers which bind non-specifically to the cell surface (e.g. cationic ferritin binding to anionic sites on the plasmalemma) and endocytosis of ligands mediated via binding to specific receptors on the cell membrane (i.e. receptor-mediated endocytes. Hepatic receptor systems have been described for the uptake of glycoproteins /65/ with *N*-acetyl galactosamine/galactose, mannose/*N*-acetyl glucosamine, mannose 6-phosphate and fucose as terminal residues /66/.

The isolated perfused liver system provides an ideal model to study the gross kinetics of uptake from the perfusate. In particular, ligands or reagents can be recirculated then removed from the perfusion medium to establish a "pulse" and replaced by fresh medium to provide a "chase"; the temperature of the perfusion medium can be rapidly switched from one level to another over a wide range (4-37°) allowing synchronized ligand entry /67/. Other useful protocols include the measurement of first-pass uptake, competition between ligands (Fig. 7) and perturbation of endocytosis after chemical challenge (Fig. 8). Although the use of specific inhibitors may assist in unraveling the mechanism(s) of uptake, the consequences of inhibited receptor-

mediated endocytosis /62/ to liver and whole body functions are not understood. Nevertheless, the process is the summation of a number of complex molecular and cellular events including recognition, internalisation and receptor recycling and in our experience the rate of uptake of macromolecular ligands is a useful means of assessing liver viability. For example, the clearance rate of saturating levels of ^{125}I-labelled asialoglycoprotein *in vivo* and in the perfused liver /67/ are similar at approximately 5μg/g liver/min, whereas with rat hepatocytes uptake is considerably less and ranges between 0.1 and 0.6 ug/g/per min.

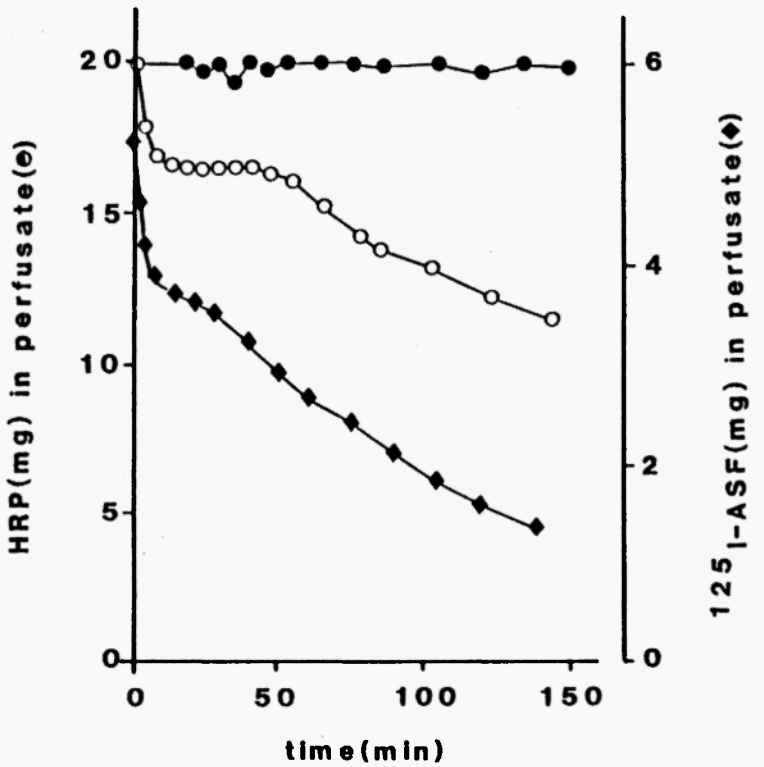

Fig. 7: Inhibitory effect of ^{125}I-asialofetuin on the uptake of HRP by the isolated perfused rat liver. The figure shows two separate experiments one in which HRP (20mg) only was added to the perfusate at time zero (○) and the other showing the inhibition of HRP (20mg) uptake (●) in the presence of 5.2ug of ^{125}I-asialofetuin (◆).

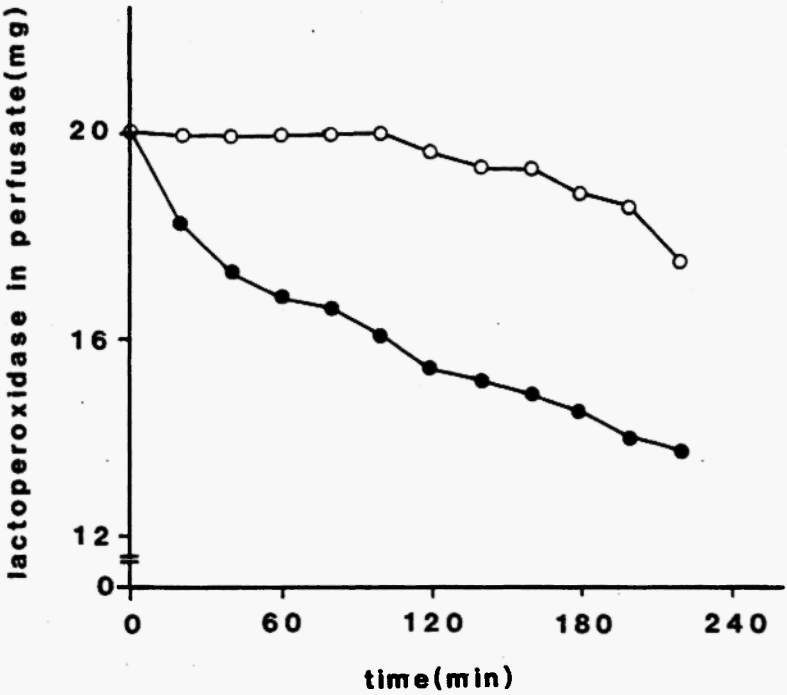

Fig. 8: The uptake of lactoperoxidaase in the absence (●) and presence of 0.8mM dansylcadaverine (○).

Endocytosis in the perfusion system also appears to be particularly sensitive to changes in the composition and physical state of the perfusate /67/. Furthermore, chemicals which perturb receptor-mediated endocytosis by modifying the plasma membrane may also interfere with cell-cell interactions and these can be studied in viable isolated organ systems.

Collectively these techniques for measuring quantitatively the production and uptake of macromolecules are used (in these laboratories) as important viability tests of hepatic function. Moreover, it is on the basis of this background information that metabolic and toxicity studies are undertaken with confidence in the isolated perfused liver system.

VII. METABOLISM OF EXOGENOUS COMPOUNDS IN THE ISOLATED PERFUSED LIVER

The use of the isolated perfused liver in metabolic studies of foreign compounds and normal biochemicals has many advantages all of which stem from the fact that normal hepatic architecture is maintained /68,69/. Metabolic capabilities can be realistically assessed because cell-cell interactions and intracellular architecture are preserved and the relationships between substrate transport, intracellular disposition and substrate access to enzyme systems are maintained. Phase I and Phase II reactions of foreign compound metabolism have been extensively studied /70,71,72,73/ and the evidence is overwhelming that the isolated perfused liver can be used with the confident expectation that the results coincide very closely with the situation *in vivo* /57,74,75,76,77,78,79,80/. It also has the considerable advantage that the partitioning of compounds between perfusate, liver and bile /81/ can be conveniently studied in detail (Fig. 9). Suitably designed experiments can demonstrate, for example, the fate of compounds formed in the liver and whether they are destined *in vivo* for biliary excretion /82,83/ or whether by passing into perfusate they are subject to possible further metabolism by organs other than the liver.

First-pass hepatic metabolism is also readily assessed /84/ and by using various doses of the compound under investigation, the capacity of the organ can be calculated, providing valuable information on the likelihood of extra-hepatic exposure to the compound and/or its metabolites. In this connection, the system is also particularly valuable in determining the combined metabolic potential of the entire portal system. Compounds can be administered via the gut lumen in the perfused gut system and perfusate, collected under first-pass conditions can be used for isolated perfused liver studies. Analysis of perfusate emerging from the isolated gut and the isolated liver provides information on the individual metabolic contributions of both gut and liver and once more the extent of extrahepatic exposure to compounds delivered via the gut lumen can be gauged /85/.

Many workers requiring significant amounts of foreign compound metabolites for structural analysis can testify to the value of the isolated perfused liver as a metabolite factory. Metabolites can be produced in suitable amounts by delivering successive bolus doses of the parent compound or by delivering the compound by continuous

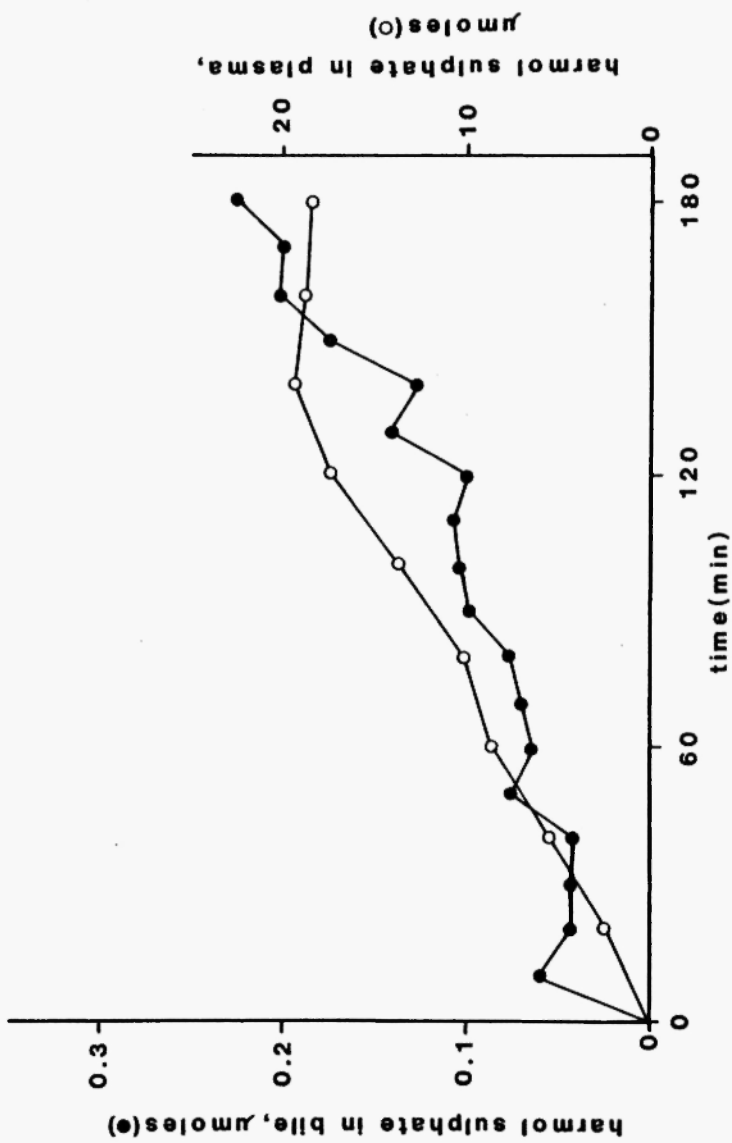

Fig. 9: The secretion of harmol ^{35}S-sulphate into bile (●) and plasma (○) by an isolated liver receiving a constant infusion of $Na_2{}^{35}SO_4$ (0.38 umoles/min) and harmol (0.35 umoles/min).

infusion. This practice is often essential in the case of toxic compounds which can be administered in small amounts only, to intact animals. Depending on the nature of the compound, its metabolites and their disposition, the bile, perfusate or the liver can provide substantial amounts of metabolites with relative ease.

It is well recognised that one of the most important criteria of an acceptable isolated perfused liver preparation is the production of bile Many workers have claimed that bile production is continuous and "normal" in perfused preparations, but few have measured bile production under sufficiently stringent conditions and at suitable time intervals over extended periods. Under the conditions described in this review the flow of bile is certainly continuous but the rate of bile formation does change during the perfusion period. There is an initial phase when flow reaches a maximum and thereafter declines and a steady-state of production is usually established. This level tends to vary from one preparation to another /51/ and also depends on the compounds under investigation /86/. Studies in these laboratories suggest that the initial phase of high bile production may be due to the perturbation of the bile production mechanism during the surgical removal of the liver /51/. However, the concentrations and absolute amounts of many choleophiles correspond with those recorded in the intact animal /87/. Reduced bile volumes are associated with increased concentrations of choleophiles while their amounts remain the same /86/.

Confidence in the isolated perfused liver system as a method for studying bile flow, the mechanisms of bile formation and the biliary excretion of compounds and their metabolites is enhanced by the knowledge that choleretic and cholestatic agents produce their known *in vivo* effects in the isolated system /88,89/. In addition, the sensitivity of bile flow to temperature change is well illustrated in the system.

Differences in the metabolism of compounds, attributable to sex and species may also be effectively studied /84,90/. Although the isolated perfused rat liver is the most studied system, in these laboratories the guinea pig liver has been used effectively to study steroid metabolism /84/. The results of these investigations when compared with results obtained with rat liver have highlighted not only the importance of sex as a metabolic determinant, but also the pitfalls of using particular solvents as vehicles for the compound under investigation. For example polyethylene glycol (PEG) 200 is not an inert solvent either in the

guinea pig or in the rat. It is sulphated particularly in the guinea pig /90/ and therefore when available SO_4^{2-} is limited, the metabolism and transport of other compounds might be affected.

The role of the isolated liver as a "metabolite factory" has already been emphasised, but a specialised aspect of this role is exemplified by the use of the system in the preparation and identification of sulphate conjugates. These are notoriously difficult to prepare and identify, but the isolated liver system has great potential here. In these laboratories we have shown that conjugates can be prepared and identified by allowing foreign compound metabolism in the presence of inorganic ^{35}S-sulphate.

An effective example is given by a study of Thymoxamine (opilon) metabolism (unpublished data, this laboratory). The suspicion that sulphate conjugates were present in bile was confirmed in experiments in which the isolated liver preparation received a continuous infusion of inorganic ^{35}S-sulphate. When the steady state of ^{35}S-excretion in bile had been established unlabelled Thymoxamine was administered as a single bolus. Over the following 30min the ^{35}S content of the bile increase approximately 6-fold. Subsequent analysis of bile samples demonstrated that some of the ^{14}C-metabolites were sulphoconjugates (see Fig.10).

VIII. DISCUSSION

In common with other experimental techniques, the state of the art is variable from one laboratory to another and the chosen experimental conditions are often biased depending on what questions are being asked.

One of the most striking features of the isolated perfused liver technique that emerges from scrutiny of the literature and is amply demonstrated from our own extensive studies, is the ability of the organ to perform some normal hepatic functions under a wide variety of experimental conditions. Less stringent conditions seem to be necessary when the liver is used as a "metabolite factory" and the liver is able to metabolise a wide range of xenobiotics and anutrients under conditions that are far removed from those operating *in vivo*. For this reason, the isolated perfused liver system has proven ideal for drug metabolism studies, but its very robustness means that other parameters must be used as sensitive criteria of assessment of change in the

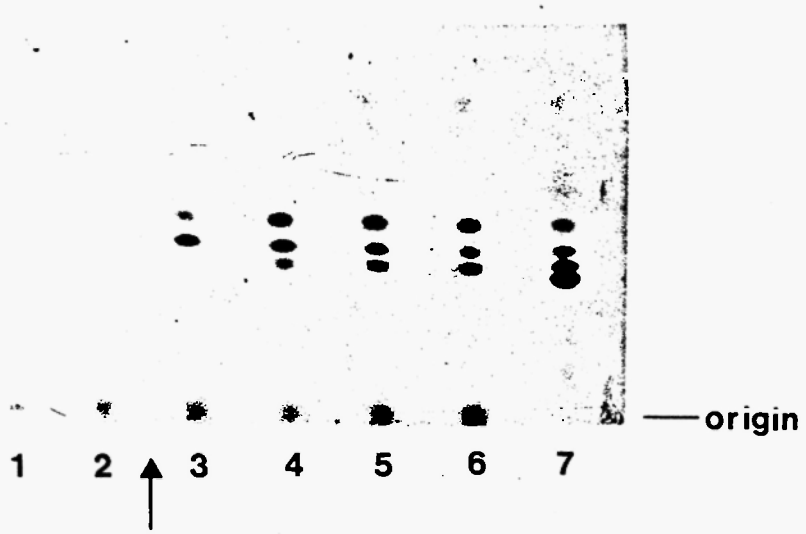

Fig. 10: Autoradiogram of a thin-layer chromatogram of bile samples (1-6) obtained from an isolated perfused rat liver receiving a continuous infusion of inorganic ^{35}S-sulphate. Samples 1 and 2 were obtained at 60min and 80min respectively and samples 3,4,5 and 6 at 20 min intervals following the addition of 8mg of Thymoxamine (Opilon) aded to the perfusate after 90 min. Sample 7 was bile obtained from a rat receiving ^{14}C-labelled Thymoxamine.

presence of toxins. The more metabolically demanding hepatic processes, in terms of the supplies of energy, substrates and cell integrity, have been studied in detail and the preparation has been optimized in terms of the rates of production of normal biochemicals. Under these optimized conditions, the performance of the isolated perfused liver is comparable with that of the organ *in vivo* with respect to such features as the kinetics of uptake from the perfusate, intra-organ distribution partition of products between perfusate and bile, biliary excretion kinetics, output of protein, glycoprotein and lipoprotein as well as receptor-mediated endocytosis. Comparable functions of the liver *in vivo* and our isolated perfused liver system contrast sharply with the data obtained with isolated hepatocyte preparations. Observations made during these studies highlight the importance of the careful

consideration required in choosing an appropriate model system in which to study toxic effects. In hepatocyte preparations there is great variation (greater than 10-fold) from one preparation to another in, for example, the levels of incorporation of radiolabelled precursors into protein and the uptake of radiolabelled glycoproteins Moreover hepatocyte preparations with low biosynthetic activity are more susceptible to toxic insult. Our work demonstrates quite clearly that hepatocyte preparations can be very misleading in toxicity testing because of the increased sensitivity of compromised cells. These problems do not arise in the isolated perfused liver system because it mimics the *in vivo* situation very closely and the viability assessments are remarkably constant from one liver to another.

The rationale for choosing macromolecular production and uptake appears to be justified in that, for particular toxins it identifies those areas of normal biochemistry which are affected and eliminates other areas which are not involved. The system has been investigated with a wide range of known hepatotoxins and yet some of these appear to have no effect on any of the parameters used routinely for toxicity testing both in the short term (0-6h) and following pretreatment of donor animals over several months. There are two possible explanations for nil effect, firstly the toxins (or their metabolites) have genuinely no effect on the synthesis/secretion/uptake of macromolecules or that the compensating capacity of the liver overcomes the effect. The first explanation is by far the most likely because known inhibitors of uptake and secretion are very effective in the isolated perfused liver

Knowledge acquired in these laboratories over a number of years has been extremely valuable and perhaps its major contribution has been to extend significantly the isolated perfused liver system for the testing of hepatotoxins. These advances have allowed us to differentiate between whole areas of hepatic function and to identify those areas affected by particular toxins. The way forward must be to build on these methodologies and current work is directed towards more precise localisation of initial toxin-induced aberrations in liver biochemistry. Our current approach is to use the proven isolated perfused liver model in conjunction with rapidly developing new technology in non-invasive physico-chemical techniques. The major objectives are to measure intracellular events and changes in the heterogenous microenvironments on the cell surface in response to toxins. Nuclear magnetic resonance techniques with perfused livers have already given some indication of

what can be achieved /91,92/. However, with improved sensitivity of NMR at the tissue level in combination with the more sensitive ESR techniques and biosensors, it is envisaged that the acquisition of much new information will be possible. By extending the perfusion model to include liver damage following ischaemia, onset of jaundice, diabetes infections, hepatomas and iatrogenic diseases, it will be possible to compare certain aspects of intermediary metabolism in normal and abnormal livers as well as to pinpoint perturbations caused by hepatotoxins.

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