

An Optimized Model for Rat Liver Perfusion Studies

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Conditions which influence the viability, integrity, and extraction efficiency of the isolated perfused rat liver were examined to establish optimal conditions for subsequent work in reperfusion injury studies including the choice of buffer, use of oncotic agents, hematocrit, perfusion flow rate, and pressure. Rat livers were perfused with MOPS-buffered Ringer solution with or without erythrocytes. Perfusates were collected and analyzed for blood gases, electrolytes, enzymes, radioactivity in MID studies, and lignocaine in extraction studies. Liver tissue was sampled for histological examinations, and wet:dry weight of the liver was also determined. MOPS-buffered Ringer solution was found to be superior to Krebs bicarbonate buffer, in terms of pH control and buffering capacity, especially during any prolonged period of liver perfusion. A pH of 7.2 is chosen for perfusion since this is the physiological pH of the portal blood. The presence of albumin was important as an oncotic agent, particularly when erythrocytes were used in the perfusate. Perfusion pressure, resistance, and vascular volume are flow-dependent and the inclusion of erythrocytes in the perfusate substantially altered the flow characteristics for perfusion pressure and resistance but not vascular volume. Lignocaine extraction was relatively flow-independent. Perfusion injury as defined by enzyme release and tissue fine structure was closely related to the supply of O₂. The optimal conditions for liver perfusion depend upon an adequate supply of oxygen. This can be achieved by using either erythrocyte-free perfusate at a flow rate greater than 6 ml/min/g liver or a 20% erythrocyte-containing perfusate at 2 ml/min/g. © 1996 Academic Press, Inc.

INTRODUCTION

The isolated perfused rat liver is a widely used model for simulating *in vivo* conditions, with the advantage of being able to precisely control experimental conditions. However, the optimal conditions for perfusion research remain ill defined in terms of such considerations as the inclusion of erythrocytes in the perfusing solutions [1], appropriate flow rates, buffer composition, and the

preferred concentration of albumin [2, 3]. While most studies report O₂ consumption and bile flow, other measures of liver function are less well defined and in many cases the viability of the preparations used is not well established.

In this study, we have examined the conditions which influence the viability, integrity, and extraction efficiency of the isolated perfused rat liver. The key conditions examined were the nature of the buffer, the presence of erythrocytes and albumin, and perfusate flow rate. Tissue function and viability have been assessed by many separate procedures including histological examination of tissues, O₂ consumption and bile flow, wet:dry tissue weight ratios, enzyme release, perfusion pressure, multiple indicator dilution (MID) studies, and lignocaine extraction.

MATERIALS AND METHODS

Animals and surgery. Female Sprague-Dawley rats weighing between 200 and 275 g were obtained from the Medical School, University of Queensland. All animal studies received ethical approval. Animals were fasted for 24 hr before experimentation. Anesthesia consisted of a single intraperitoneal injection of a mixture of 2.5 mg xylazine, 12.5 mg ketamine, and 0.5 mg diazepam in a total volume of 350 µl. Five hundred units of heparin was injected into the inferior vena cava to prevent blood clotting. The portal vein was cannulated with a 16-gauge iv catheter and the liver perfused with MOPS-buffered Ringer solution at a flow rate of 10 ml/min. The abdominal inferior vena cava was cut quickly and the flow rate increased to 0.05–0.15 ml/min/g body weight. The chest of the animal was opened and the inferior vena cava was cannulated via the right atrium of the heart using a 10-cm length of polyethylene tubing (Intramedic, Clay Adams, Parsippany, NJ; PE-240, i.d. 1.67 mm). The abdominal inferior vena cava was ligated and the perfusate outflow was drained to a waste bottle, thereby flushing the blood from the liver. The bile duct was also cannulated using a 5-cm length of polyethylene tubing (PE-10, i.d. 0.28 mm). Bile was collected into Eppendorf tubes. Liver wet weight ranged from 4.6 to 6.8 g and averaged 5.7 ± 0.6 g (mean \pm SEM). Erythrocytes were collected from domestic dogs. The dogs were being destroyed under guidelines approved by the University of Queensland Animal Experimentation Ethics Committee, and the collection procedure was approved by the same committee. Blood was collected directly into collection bags containing citrate (citric acid 3.27 g/liter; sodium citrate 26.30 g/liter; Tuta Laboratories, Lane Cove, NSW, Australia) by cardiac puncture. The dog blood was washed three times in phosphate-buffered saline, with the buffy coat removed after each wash. The washed erythrocytes were then further washed with three

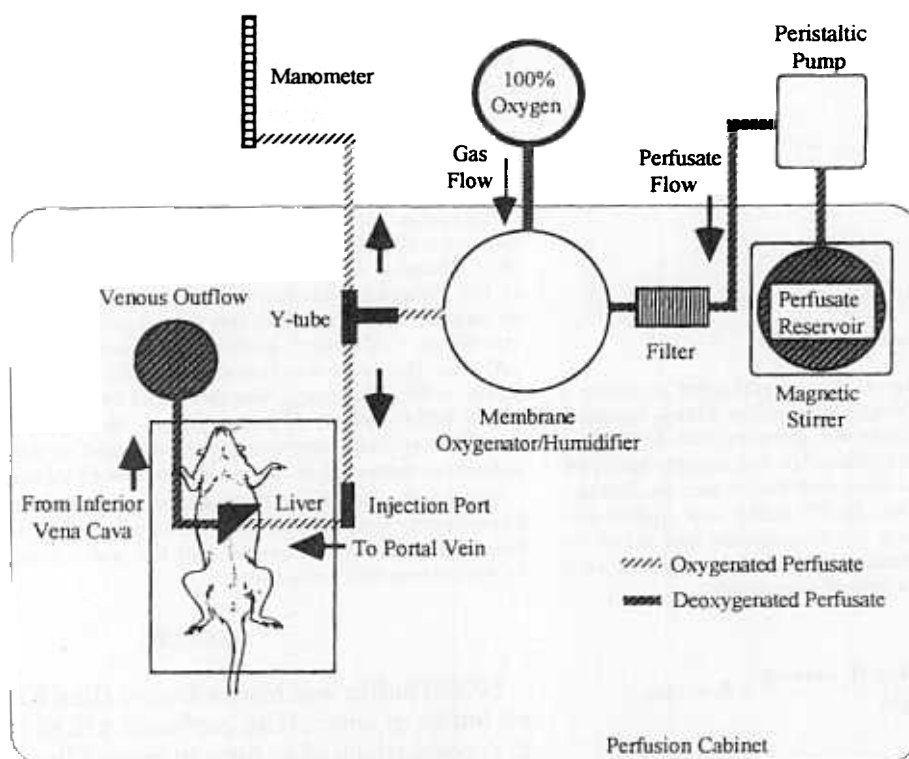


FIG. 1. Semidiagrammatic view of the perfusion system. Direction of flow is indicated by arrows.

changes of MOPS buffer (3-[N-morpholino]propanesulfonic acid; Sigma, St. Louis, MO) containing 2% bovine serum albumin (BSA). The pH of the MOPS buffer was carefully adjusted with NaOH so as to achieve the desired pH of 7.2 after the 20% (v/v) erythrocytes were added to the buffer. The hematocrit of the final suspension was determined to a value of $15.4 \pm 1.6\%$.

Buffers. Perfusion media investigated were Krebs-Henseleit buffer and MOPS-buffered Ringer solution. Both the Krebs-Henseleit buffer [4] and the MOPS-buffered Ringer solution [5] contained 11.1 mM glucose, with or without 2% dialyzed BSA (Sigma; bovine albumin fraction V, Cat. No. A-7906). Dialysis tubing was treated with sodium bicarbonate and EDTA before use. Forty grams of BSA was dissolved in 400 ml of MOPS buffer (25 mM) and dialyzed for 48 hr with two changes of 4 liters of MOPS buffer. Preliminary studies showed that dialyzed BSA effectively reduced hemolysis of dog erythrocytes during liver perfusion studies. The pH of rat portal venous blood *in vivo* was measured as 7.209 ± 0.005 ($n = 4$) and all studies were therefore performed at pH 7.2. The pH of the Krebs-Henseleit buffer was adjusted by the amount of NaHCO_3 added. The pH of the MOPS-buffered Ringer solution was adjusted with 5 N NaOH, before addition of erythrocytes as described above. Prior to experiments commencing, all buffer solutions (with or without BSA) were filtered through a 5- μm membrane filter (Millipore, Maidstone, England) to remove any potential microemboli [6]. The osmolality of the MOPS buffer used was 288 ± 8 mOsm/kg.

Perfusion system. The buffer was drawn from its reservoir by a peristaltic pump (Masterflex; Cole-Parmer Instrument Co, Chicago, IL) and passed in sequence through a bubble trap with in-line filter, a membrane oxygenator comprising 4 m of coiled silastic tubing (Dow Corning Co, Midland, Michigan; i.d. 1.5 mm), a Y-tube connected to a manometer, and eventually into the portal vein of the animal through a Teflon cannula (Fig. 1). The inclusion of an injection port positioned immediately prior to the portal vein cannula allowed collection of portal inflow samples. Outflow samples were collected via the inferior vena cava cannula. The perfusate was stored in a glass reservoir standing on a magnetic stirrer. The perfusion system was housed in a perfusion cabinet with temperature and humidity maintained by a water bath underneath.

The membrane oxygenator generated a partial pressure of O_2 of 576 ± 9 mm Hg ($n = 16$). We found that the $p\text{O}_2$ of the inflow samples was greatly affected by the mode of specimen collection. If the sample was collected by a plastic syringe inserted into the injection port, the $p\text{O}_2$ obtained would be about 50–100 mm Hg lower than that collected by just allowing the perfusate to drip from the port into a collection vial which was then sealed before $p\text{O}_2$ measurement. This discrepancy is probably a result of gas exchange turbulence occurring when perfusate was drawn into the syringe. Laboratory workers unaware of this discrepancy could have reported underestimated $p\text{O}_2$ s and consequently undervalued O_2 supply and O_2 consumption (if the inflow sample is taken by syringe and outflow sample by dripping).

The outflow perfusate was recycled to the central reservoir for recirculation, except when MID or lignocaine extraction studies were performed. In these studies a single-pass perfusion design was employed in which a bolus of an indicator or lignocaine solution was injected into the portal vein and the outflow collected for analysis. Flow rate was regulated by a variable speed pump drive (Masterflex) and maintained constant throughout an experiment. The actual rate was determined manually using measuring cylinder and timer.

Histological studies. At the conclusion of each experiment, the liver was completely excised and freed of any extraneous tissue. Multiple small samples were taken from each liver (mean 6.3 mm, range 2–9 mm) and placed in 10% neutral buffered formalin (SEA Trading, Brisbane, Australia) for histological examination. Sections were examined blind and the extents of apoptosis and necrosis graded separately (0 to 3+). The remaining liver was carefully blotted and the wet weight determined. The liver was then placed in an oven for 24 hr at 80°C , after which time the dry weight was determined, and the wet:dry weight ratio was calculated.

Biochemical studies. pH, $p\text{O}_2$, $p\text{CO}_2$, and hematocrit of the collected perfusate were determined with an ABL 520 analyzer (Radiometer, Copenhagen, Denmark) and total hemoglobin and O_2 content (vol%) were measured with an OSM3 analyzer (Radiometer) operating in the dog mode. Hepatic oxygen consumption ($\mu\text{mol}/\text{min}/\text{g}$ liver) was determined by the formula

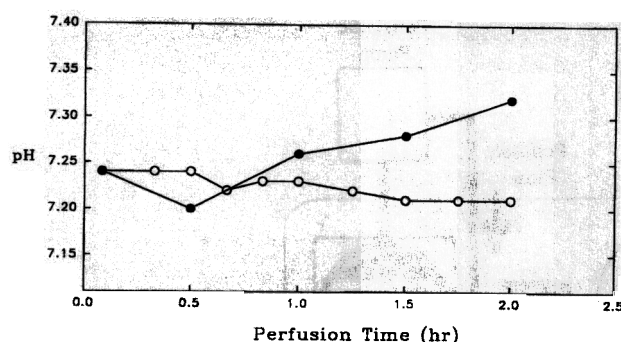


FIG. 2. Changes in perfusate pH during prolonged perfusion of rat livers with MOPS-buffered Ringer solution or Krebs-Henseleit bicarbonate buffer. Isolated rat liver was perfused with MOPS-buffered Ringer solution (○) or Krebs-Henseleit bicarbonate buffer (●) for a period of 2 hr. The Krebs-Henseleit buffer was equilibrated with 5% CO₂ and 95% O₂ and the MOPS buffer was equilibrated with 100% O₂. The perfusate was not recirculated and pH of the outflow from the liver was determined at regular intervals. Data shown were obtained from single typical experiment.

$$\frac{\text{inflow O}_2 \text{ content} - \text{outflow O}_2 \text{ content}}{\text{liver wet weight}} \times \text{flow rate,}$$

and oxygen supply ($\mu\text{mol}/\text{min}/\text{g}$ liver) was determined by the formula

$$\frac{\text{inflow O}_2 \text{ content}}{\text{liver wet weight}} \times \text{flow rate.}$$

Sodium, potassium, aspartate transaminase (AST), and lactate dehydrogenase (LD) were determined with a Hitachi 747 analyzer (Hitachi, Tokyo, Japan). Hemolytic index, which indicates the extent of hemolysis, is automatically calculated on the Hitachi 747 from differential wavelength readings at 408, 505, 570, and 600 nm, and was routinely determined on all samples containing dog erythrocytes. (A hemolytic index of 100 equates to approximately 1 g of free hemoglobin per liter of solution.) Because LD may be released from either erythrocytes or hepatocytes, the hemolytic index was an integral part of determining the source of any increases in LD activity. Where necessary, the source of LD was followed up by isoenzyme fractionation on agarose gel [7].

Multiple indicator dilution studies. MID technique used in this study essentially follows the methodology utilized in previous studies [3]. The injectate used to define the vascular, extracellular, and total water volumes consisted of [³H]H₂O (1 μCi), [¹⁴C]sucrose (0.2 μCi), and [¹²⁵I]-BSA (2 μCi) in 50 μl of perfusate. The injectates were delivered as rapid boluses via the inflow catheter, and their effluent was collected at 1- to 2.5-sec intervals over the initial 70 sec. All samples were stored at 4°C prior to analysis. All outflow samples and appropriate injectate dilutions were centrifuged and 100 μl of supernatant was transferred to an Eppendorf tube. Two hundred microliters of acetonitrile was added and all samples were centrifuged again, prior to samples being taken for counting of gamma radiation (Packard Cobra III gamma counter; Downers Grove, IL) and beta radiation (1600TR series liquid scintillation counter; Packard). The outflow concentration for each solute at each collection time was expressed as a fraction of the injected dose per milliliter in the perfusate eluting at the midpoint of the collection interval. The total recovery of all solutes was assessed from the area under the outflow concentration-time profile (AUC), the measured perfusate flow rate, and the amount of marker injected (Dose), using the trapezoidal rule for integration:

$$\% \text{ recovery} = \frac{\text{AUC}}{\text{Dose}} \times \text{flow rate.}$$

The mean transit time (MTT) for each solute was calculated from the ratio of the area under the first moment curve, i.e., outflow concentration time-time profile (AUMC) to the area under the curve (AUC), i.e., $\text{MTT} = \text{AUMC}/\text{AUC}$. The volume of distribution for each marker was then estimated from the product of MTT and flow rate [8]. The resistance was estimated as the observed perfusion pressure corrected for catheter effects divided by the observed flow rate.

Lignocaine extraction. Lignocaine extraction across the liver was used as a viability measure for the efficiency of hepatic metabolism [9]. Lignocaine was given as a bolus injection (approximately 20 μg in 100 μl) and the outflow from the liver collected for 5 min. A 10-ml sample was taken from this pooled collection and lignocaine extracted on 1-ml phenyl bond-elute columns (Varian, Harbor City, CA). The lignocaine was eluted with 17.5% (v/v) acetonitrile in phosphate buffer. Lignocaine was measured by injection onto a reverse-phase HPLC system (Waters, Milford, MA) with detection at 210 nm. The lignocaine extraction was estimated as the total amount of lignocaine recovered in the perfusate divided by the dose injected.

Statistical analysis. All values are expressed as means \pm SEM. Experiments were carried out in triplicate unless stated otherwise. Results were considered significant if P was <0.05 when compared by ANOVA or Student's t test.

RESULTS

MOPS buffer was more effective than Krebs-Henseleit buffer at controlling perfusate pH as shown in Fig. 2. A comparison of various indices of liver function between the two buffer systems is shown in Table 1. The only significant difference was in the wet:dry weight ratios, which were significantly lower when MOPS buffer was used.

In the absence of albumin, wet:dry weight ratios of livers after 3 hr of perfusion were significantly higher than in the presence of undialyzed albumin (3.92 ± 0.04 versus 3.23 ± 0.04 ; $P < 0.001$), indicating the development of significant tissue edema in the former. Hemolytic indices of the perfusate during a 2-hr perfusion were also much higher in the absence of albumin. Dialysis of the albumin before use resulted in a further lowering of the hemolytic index as shown in Fig. 3. It should be noted that these differences in hemolytic index are associated with significant LD release (>50 U/liter) although no hemolysis is apparent on naked eye examination.

TABLE 1

Comparative Indices of Liver Function in Livers Perfused with either MOPS or Krebs-Henseleit Buffers

Parameter	MOPS buffer	Krebs-Henseleit
Bile flow ($\mu\text{l}/\text{min}/\text{g}$)	0.62 ± 0.03	0.57 ± 0.06
O ₂ consumption ($\mu\text{mol}/\text{min}/\text{g}$)	1.9 ± 0.1	1.8 ± 0.03
LD release (U/g/120 min)	47.5 ± 7.3	72.4 ± 18.4
AST release (U/g/120 min)	5.9 ± 1.6	10.3 ± 2.6
Wet:dry weight ratio	$3.6 \pm 0.2^*$	$4.0 \pm 0.1^*$

Note. Rat livers were perfused at 3.5–4.5 ml/min/g with the erythrocyte-free buffers which contained 2% BSA. The livers were removed for weight determination at the end of the perfusion. The values are expressed as means \pm SEM ($n = 4$ for MOPS and $n = 3$ for Krebs-Henseleit).

* $P < 0.05$, unpaired t test.

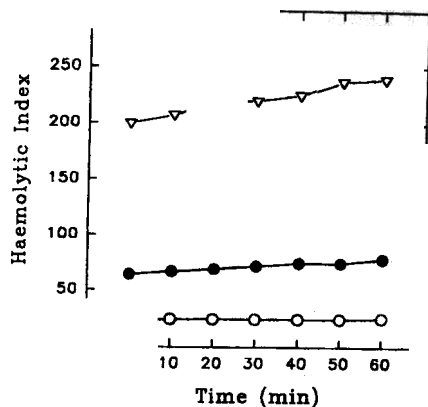


FIG. 3. Effect of albumin and dialysis upon the hemolytic index. MOPS-buffered Ringer solution containing no BSA (∇), 2% undialyzed BSA (\bullet), or 2% dialyzed BSA (\circ) was pumped through the perfusion system without the rat liver for a period of 60 min. The buffer was sampled at regular intervals for the determination of hemolytic index. Data shown were obtained from single typical experiment.

The effect of O_2 supply and O_2 consumption on various parameters of liver function is shown in Figs. 4 and 5. From Fig. 4 it is apparent that bile flow is very dependent upon O_2 supply until a critical value of approximately $3.5 \mu\text{mol/min/g}$ of liver is reached, for perfusion in the presence or absence of erythrocytes. At this oxygen supply, the bile flow was $0.93 \pm 0.06 \mu\text{l/min/g}$ liver measured at 1 hr after onset of perfusion and $0.72 \pm 0.06 \mu\text{l/min/g}$ at 2 hr. The average bile flow over the 2-hr perfusion period was $0.82 \pm 0.10 \mu\text{l/min/g}$.

Similarly, wet:dry weight ratios are higher at O_2 supply values below $3.5 \mu\text{mol/min/g}$ liver for perfusion in the absence of erythrocytes, though the differences are

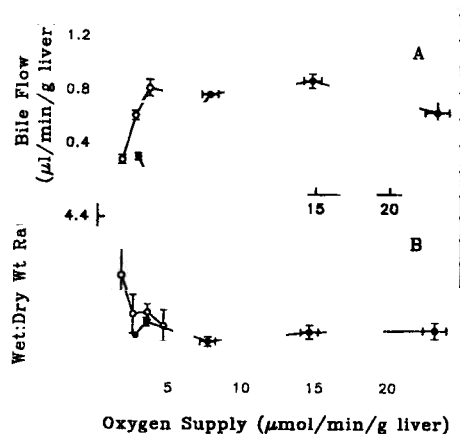


FIG. 4. Dependence of bile flow and wet:dry tissue weight ratio on oxygen supply. (A) Change in bile flow and (B) change in wet:dry weight ratio as a function of oxygen supply. Isolated rat livers were perfused with MOPS-buffered Ringer solution with (\bullet) or without (\circ) dog erythrocytes for 2 hr. The different levels of oxygen supply determined were a result of perfusing the livers of different animals at different flow rates or different hematocrits. Data are plotted as means \pm SEM from a group of three animals at each value of oxygen supply indicated.

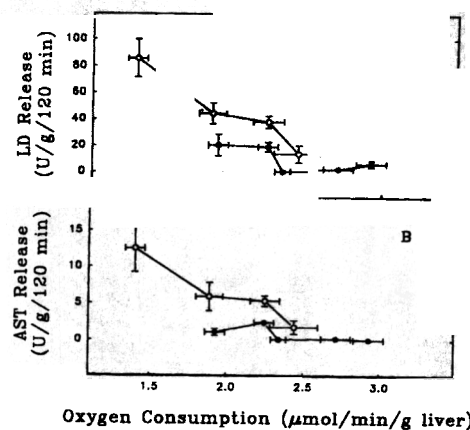


FIG. 5. Dependence of enzyme release on O_2 consumption. (A) Release of LD and (B) release of AST from perfused rat livers as a function of O_2 consumption. Isolated rat livers were perfused with MOPS-buffered Ringer solution with (\bullet) or without (\circ) dog erythrocytes for 2 hr. Data are plotted as means \pm SEM from a group of three animals at each value of O_2 consumption indicated.

not statistically significant (at O_2 supply of $1.7 \mu\text{mol/min/g}$ the wet:dry weight ratio was 3.87 ± 0.25 , and at O_2 supply of $4.7 \mu\text{mol/min/g}$ the ratio was 3.43 ± 0.13 ; $P > 0.2$). For perfusion in the presence of erythrocytes, the wet:dry weight ratio stayed low at approximately 3.3–3.5. An O_2 supply of $3.5 \mu\text{mol/min/g}$ of liver corresponds to an O_2 consumption of $2.3 \mu\text{mol/min/g}$ in the absence of erythrocytes and appears to be the critical O_2 requirement for healthy liver tissue from fasted animals. This is confirmed in Fig. 5 which shows the enzyme release at different O_2 consumptions, in the presence or absence of erythrocytes. Oxygen consumption was matched by adjusting flow rates. There was effectively no enzyme released when erythrocytes were present (minimum O_2 consumption possible was $1.9 \mu\text{mol/min/g}$, due to flow considerations), though enzyme release was observed in the absence of erythrocytes up to and including an O_2 consumption of $2.3 \mu\text{mol/min/g}$. When the O_2 consumption was identical ($2.3 \pm 0.1 \mu\text{mol/min/g}$), there was significantly less LD enzyme released when erythrocytes were present (in the presence of erythrocytes $19.5 \pm 3.5 \text{ U/g/120 min}$; in the absence of erythrocytes $38.3 \pm 4.3 \text{ U/g/120 min}$; $n = 3$, $P < 0.03$). Thus it appears that erythrocytes offer some protection from damage that is separate from their ability to provide adequate O_2 .

Histological assessment was performed on all livers. Necrosis occurred to varying degrees in all animals in the group with lowest oxygen consumption, but not in other groups. Compare Figs. 6a and 6b which show histology of liver after a 2-hr infusion at an O_2 consumption of $2.8 \mu\text{mol/min/g}$ liver, in the presence of albumin but without erythrocytes, and Figs. 6c and 6d which show a section of liver 2 hr after an infusion at an O_2 consumption of $1.3 \mu\text{mol/min/g}$ liver, also in the presence of albumin but in the absence of erythrocytes. In the former experiment LD release was 18.2 U/g/120 min , AST release was 2.4 U/g/120 min , and appearance on histology was normal. In the latter, LD release was

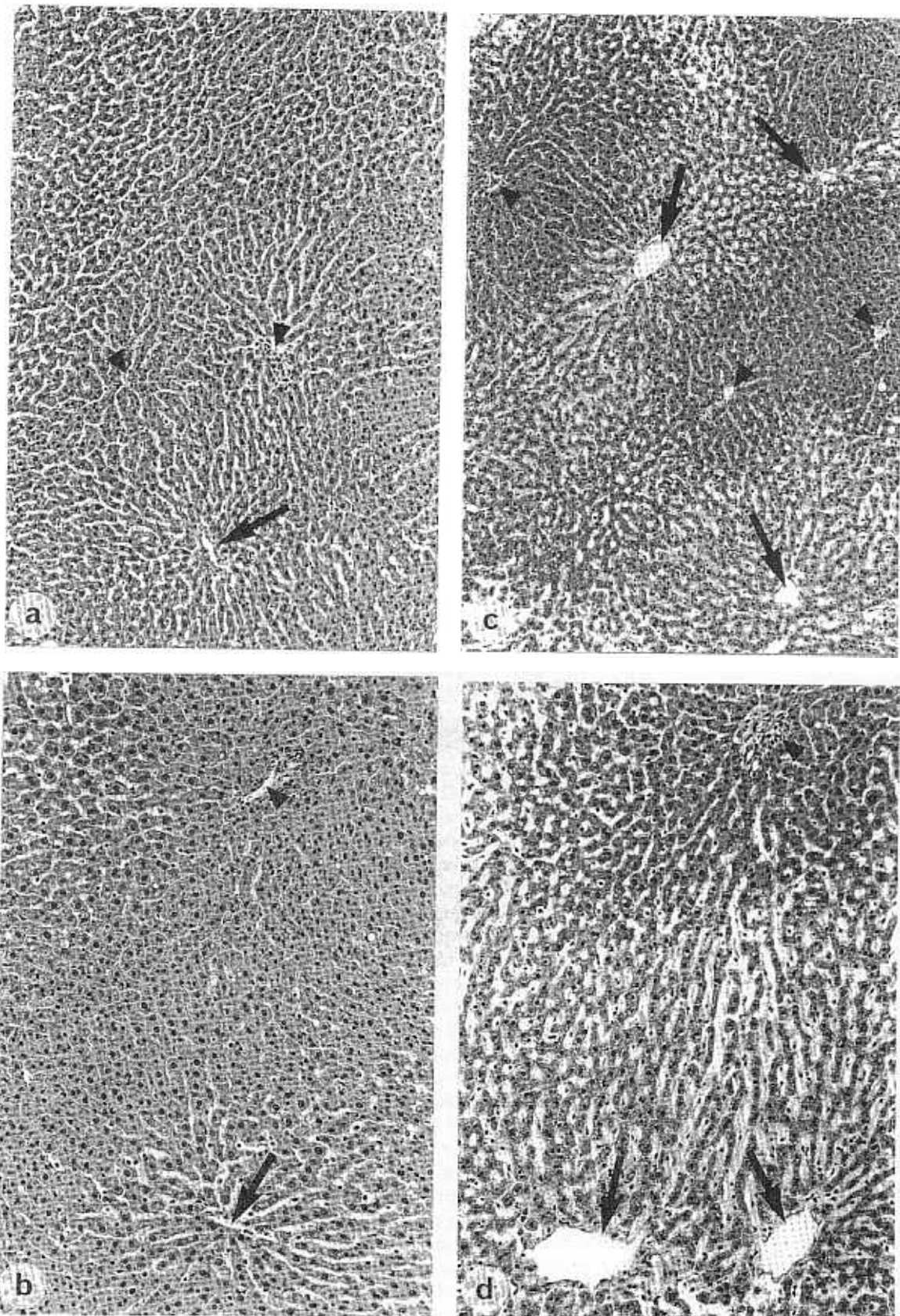


FIG. 6. Histological comparison of tissue perfused in the absence of erythrocytes, above and below the critical O_2 consumption of $2.3 \mu\text{mol/min/g}$. (a) and (b) Histologically normal liver (H&E, original magnification a $\times 130$, b $\times 165$). Oxygen consumption was $2.8 \mu\text{mol/min/g}$ liver, LD release was 18.2 U/l/120 min , and AST release was 2.4 U/l/120 min . (c) and (d) Early acinar zone 2 and 3 necrosis manifested by altered staining, altered size and shape, and fine cytoplasmic vacuolation of hepatocytes (H&E, original magnification c $\times 110$, d $\times 175$). Oxygen consumption was $1.3 \mu\text{mol/min/g}$ liver, LD release was $112.6 \text{ U/l/120 min}$, and AST release was 19.0 U/l/120 min . Arrowheads, portal tracts; arrows, terminal hepatic venules.

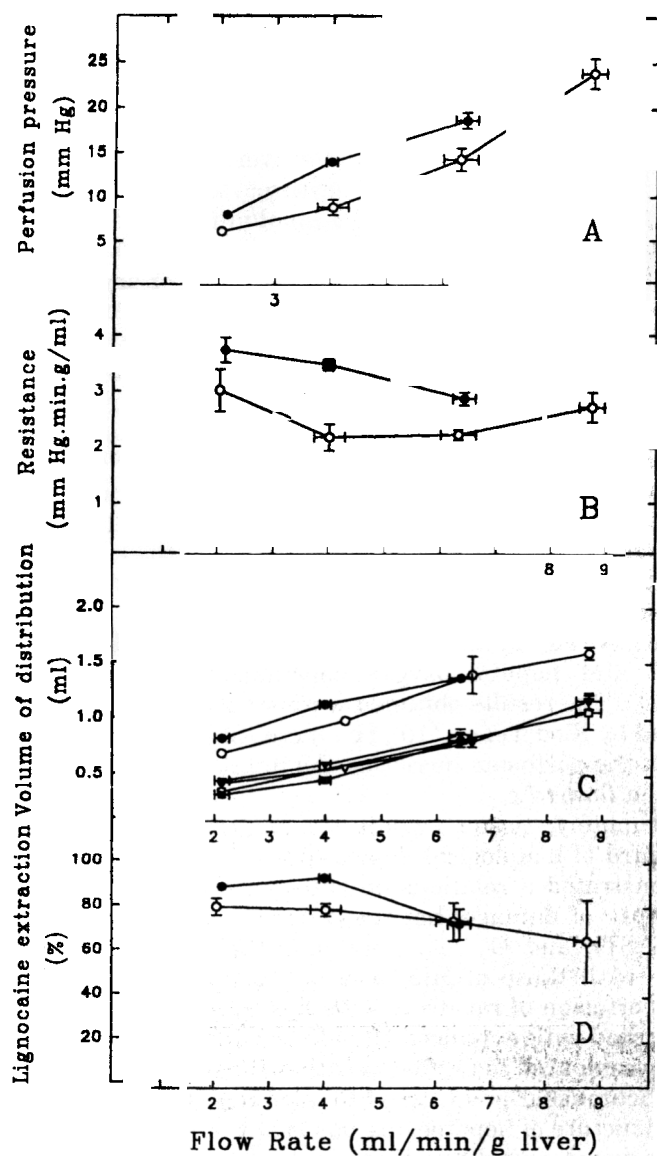


FIG. 7. Dependence of perfusion pressure, resistance, volume of distribution, and lignocaine extraction on flow rate. (A) Perfusion pressure, (B) resistance, and (D) lignocaine extraction as a function of flow rate. Isolated rat livers were perfused with MOPS-buffered Ringer solution with (●) or without (○) dog erythrocytes for 2 hr. Perfusion pressure was determined from the height of perfusate recorded on the manometer attached to the perfusion system. It is expressed in mm Hg assuming the specific gravity of perfusate is the same as water and the specific gravity of mercury is 13.534. (C) Volumes of distribution as a function of flow rate. The indicators used included [³H]H₂O in the presence (●) and absence (○) of erythrocytes, [¹⁴C]sucrose in the presence (▼) and absence (▽) of erythrocytes, and [¹²⁵I]-BSA in the presence (□) and absence (■) of erythrocytes. Flow rate was regulated by a variable speed pump drive and maintained constant throughout an experiment. Data are plotted as means ± SEM from a group of three animals at each value of flow rate indicated.

112.6 U/g/120 min, AST release was 19.0 U/g/120 min, and histology showed early acinar zone 2 and 3 necrosis, reflecting more severe cell injury at low levels of O₂ consumption.

The effect of changes in portal flow rate on hepatic circulation is shown in Fig. 7. There was no significant

change in pressure, resistance, or distribution volumes over the 2 hr of perfusion. A slight increase in pressure was observed in the third hour (not shown). There is a direct, almost linear relationship, between pressure and flow for both erythrocyte-containing and erythrocyte-free perfusate with a slightly higher pressure being observed for perfusate containing erythrocytes (Fig. 7A). As a consequence, the resistance is relatively unaffected by the flow rate used (Fig. 7B). Consistent with the flow dependence of pressure, the vascular volumes, as defined by BSA, in Fig. 7C also show an apparently linear increase with a changing flow. Figure 7C also shows that the distribution of sucrose and water in the perfused liver are linearly related to flow rate. The distribution of these solutes relative to albumin were independent of flow rate suggesting that no edema was present. Figure 7D shows that lignocaine extraction is enhanced by the presence of erythrocytes in the perfusate at flow rates less than or equal to 4 ml/min/g of tissue (greater than 90% extraction). There was no difference between the two perfusates at a flow rate of 6 ml/min.

DISCUSSION

The isolated perfused liver has a limited viability even when studies are performed under optimal conditions. Under usual conditions it may be maintained for approximately 3–4 hr [2, 3]. Nevertheless, if meaningful biological questions are to be posed and answered, as near optimal conditions as possible must be utilized. It must also be remembered that damage to the liver may not manifest for some time (for example, enzyme release from an anoxic liver may not eventuate for nearly an hour after perfusion has commenced) and to be valid either studies must be performed for sufficient time for damage to be able to manifest or initial studies must demonstrate the validity of the experimental conditions.

A variety of criteria have been used to define the functioning and viability of isolated perfused rat livers. These criteria include O₂ consumption, gluconeogenesis, redox state, bile flow, gross morphological appearance of the liver, clearance of carbon particles, and drug extraction [2, 3]. While some studies have examined the role of variables such as hematocrit [10], we are not aware of any study which has attempted to interrelate liver integrity, physiology, O₂ and drug extraction efficiency, erythrocyte presence, and perfusate flow rate.

It is generally agreed that the most important agents in determining tissue viability are the perfusate composition and O₂-carrying capacity [2]. Hardison and Wood [11] suggested that bicarbonate, present in Krebs–Henseleit buffer is required to maintain the bile salt-independent fraction of bile flow. Our study, however, shows that the MOPS buffer which contains no bicarbonate is at least as effective as, if not superior to, Krebs–Henseleit buffer in supporting bile flow and other viability indices (Table 1). Gores *et al.* [2] also

suggested that the Krebs–Henseleit buffer maintains a stable pH when equilibrated with a 95% O₂:5% CO₂ atmosphere. We have found that the perfusate pH increased with time when using Krebs–Henseleit buffer (as illustrated in Fig. 2). Rat portal blood has a pH of 7.2, thus the pK_a of MOPS buffer of 7.2 makes it an ideal buffer for liver perfusion studies. We found that there was less change in pH of the perfusate pool (Fig. 2) and less tissue edema developed when MOPS was used instead of Krebs–Henseleit buffer (Table 1). Mischinger *et al.* [12] had reported falling pH over a 3-hr perfusion period with the Krebs–Henseleit buffer, whereas Schmucker and Curtis [13] found the pH of Krebs–Ringer bicarbonate perfusate to increase during aerobic perfusion. Our result indicated an initial fall in pH followed by gradual rise. Control of perfusate pH is of critical importance as it has been shown when studying the development of reperfusion injury in isolated perfused liver that changes in pH are closely related to the degree of injury observed [14]. We have chosen a pH of 7.2 for our perfusion experiments since this is the physiological pH of rat portal blood we determined. We feel that such a pH is more appropriate than a pH of 7.4 used by most workers in this field [15–17].

Isolated organs have a time-dependent tendency to absorb water, as with a relatively protein-free medium, water gradually escapes from the vascular space and interstitial edema develops [6]. In our recirculating system, we found that presence of dialyzed BSA was important. In the absence of BSA, significant edema developed as assessed by wet:dry weight ratios. In addition, significant hemolysis occurred as assessed by the hemolytic index. This is consistent with the findings of Hems *et al.* [15] who reported that omission of albumin from the perfusion medium caused gross swelling of the liver and exudation of fluid from the surface, but varying the albumin concentration between 1.5 and 6% made no difference. Rosini *et al.* [18] also found that in the absence of an oncotic agent, release of cytosolic enzymes into the perfusate increased as the oncotic pressure decreased. We had similar findings with BSA when LD and AST were measured (data not shown). Our results are at variance with those of Mischinger *et al.* [12] who found increased tissue damage in the presence of BSA. They apparently did not dialyze their albumin before use and we found that dialysis reduced the extent of hemolysis further. There is certainly batch to batch variation of albumin and failure to dialyze may have contributed to the discrepancy between results.

The values of wet:dry weight ratio obtained in the present study (Table 1, Fig. 4) compare well with those of Exton and Park [19] who found that in fasted rats the ratio was 3.78 before perfusion and 3.85 after perfusion for 2 hr. The bile flow values obtained are also comparable to those reported in the literature [2, 3]. The observed dependence of bile flow on oxygen supply (Fig. 4) confirms that reported by Schmucker and Curtis [13].

The O₂ consumption consistent with viable tissue

varies depending upon whether animals are fed or fasted. For fed animals it has been suggested that O₂ consumption should be greater than 2.0 $\mu\text{mol}/\text{min}/\text{g}$ liver weight [2]. For fasted animals O₂ consumption may be as low as 1.1 $\mu\text{mol}/\text{min}/\text{g}$ liver. Our data indicate that in the absence of erythrocytes, an O₂ consumption of approximately 2.3 $\mu\text{mol}/\text{min}/\text{g}$ liver is the critical O₂ requirement for healthy liver tissue from fasting animals. However, in the presence of erythrocytes, little enzyme release occurred at O₂ consumption as low as 1.9 $\mu\text{mol}/\text{min}/\text{g}$ liver. This study indicates that erythrocytes are doing something in addition to simply delivering O₂ and that in some manner they ameliorate the damage observed at that O₂ consumption rate. The exact nature of this beneficial effect is unknown but it has been postulated that when erythrocytes are absent from the perfusate, endothelial cells may change their shape, grow larger within the lumen, and thus create an obstacle to the free perfusion of sinusoids [20].

We have also studied the relationship between hematocrit and hepatic oxygen consumption (data not shown). The results obtained were similar to those reported by Riedel *et al.* [10], i.e., increasing the hematocrit of the perfusate increased the uptake of oxygen at a given flow rate.

Ultimately, tissue viability is judged by the gold standard of histological appearance [13, 21]. We have demonstrated a relationship between histological appearance of damage, hepatocyte enzyme release (LD and AST), and O₂ consumption rates. Our findings agree with those of Hanks *et al.* [22] who observed that perfusion of rat livers with 10% erythrocytes was associated with enhanced O₂ delivery and less cellular injury compared to perfusion with cell-free buffer solution. Schmucker and Curtis [13] also reported that the fine structure of hepatocytes was best preserved in livers perfused with dilute blood in comparison to perfusion with hemoglobin-free medium.

In addition to these basic indices of tissue viability, we have studied several other useful technical procedures in this current work. The availability of the hemolytic index is an important technical advance in studies of the perfused liver when erythrocytes are contained in the perfusate. The potential for confusion as to the source of LD is removed by use of this index. In addition, subtle changes such as those indicated above, i.e., the effect of albumin and dialysis on erythrocyte hemolysis, can only be identified by means of the hemolytic index.

The relationship between perfusion pressure and resistance with perfusate flow rate is similar to that reported by Le Couteur *et al.* [23] for erythrocyte-free perfusate. The addition of erythrocytes increases both the perfusion pressure and the resistance (Figs. 7A and 7B). The portal vein pressures observed in this work are less than the 8 to 10 mm Hg reported for the liver [2] and may result in an uneven distribution of perfusate in the liver. However, injection of Evans blue into the liver using albumin-free perfusate demonstrates an

even distribution of dye throughout the liver. Pang *et al.* [24] suggested that a flow rate of 10 ml/min of a perfusate containing 20% erythrocytes in a perfused rat liver system is required to furnish a pressure of 13 cm H₂O (10 mm Hg), the critical pressure for maintaining opening of the vasculature. A parabolic decrease in volume with flow may be observed below the critical pressure [22]. The parallel total water and vascular volume-flow rate relationships (Fig. 7C) are consistent with the known linear relationships between the hepatic blood volume and intrahepatic pressure [25] and minimal edema. A divergence in the relationships with flow rate would have been anticipated if a significant fluid shift into hepatocytes had occurred. The relatively constant wet:dry weight ratio with increasing flow rate is also consistent with minimal edema formation. Figure 7D shows that the extraction of lignocaine is dependent on erythrocyte presence at or below 4 ml/min/g flow rate. The lower extraction of lignocaine in the absence of erythrocytes is consistent with an impaired metabolism of lignocaine in the hypoxic conditions present. Pang and Rowland [9] have suggested that a high lignocaine extraction is supportive of good liver viability. Keiding *et al.* [26] have also emphasized that flow rate and hematocrit must be kept above certain limits in metabolic studies with the perfused liver.

In conclusion, the optimal conditions for liver perfusion depend upon an adequate flow rate and hematocrit to exceed the critical perfusion pressure and to provide sufficient oxygenation. Gross liver appearance, pressure and volume assessments, bile flow rate determination, enzyme release, wet:dry weight ratios, O₂ extraction, and lignocaine extraction provide evidence of physiological metabolic viability. Confirmation of inadequate conditions is provided by histological assessment. This study has shown that MOPS is superior to Krebs-Henseleit as a perfusion buffer and that the optimal condition for the use of this buffer is at an O₂ consumption exceeding 2.3 μ mol/min/g liver when livers from fasting animals are used. The O₂ consumption is achieved using either erythrocyte-free perfusate at a flow rate greater than 6 ml/min/g liver or a 20% erythrocyte-containing perfusate at 2 ml/min/g liver. It appears that if these conditions are met, all physiological, biochemical, and histological criteria for the assessment of liver viability are fulfilled.

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REFERENCES

- Ballet, F. A., and Thurman, R. G. Why the perfused liver? In F. Ballet and R. Thurman (Eds.), *Perfused Liver: Clinical and Basic Applications*. London: Libbey, 1991. Pp. 1-20.
- Gores, G. J., Kost, L. J., and Larusso, N. F. The isolated perfused rat liver: Conceptual practical considerations. *Hepatology* 6: 511, 1986.
- Wolkoff, A. W., Johansen, K. L., and Goesser, T. The isolated perfused rat liver: Preparation and application. *Anal. Biochem.* 167: 1, 1987.
- Krebs, H. A., and Henseleit, K. Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Z. Physiol. Chem.* 210: 33, 1932.
- Blanchard, J. S. Buffers for enzymes. *Methods Enzymol.* 104: 461, 1984.
- Yeh, T., and Wechsler, A. S. The isolated organ in research. In H. Troidl, W. O. Spitzer, and B. McPeck (Eds.), *Principles and Practice of Research: Strategies for Surgical Investigations*. New York: Springer-Verlag, 1991. Pp. 354-365.
- Preston, J. A., Briere, R. O., and Batsaker, J. A. Rapid electrophoretic separation of lactate dehydrogenase isoenzymes on cellulose acetate. *Am. J. Clin. Pathol.* 43: 256, 1965.
- Roberts, M. S., Fraser, S., Wagner, A., and McLeod, L. Residence time distributions of solutes in the perfused rat liver using a dispersion model of hepatic elimination. I. Effect of changes in perfusate flow and albumin concentration on sucrose and taurocholate. *J. Pharmacokinetic. Biopharm.* 18: 209, 1990.
- Pang, K. S., and Rowland, M. Hepatic clearance of drugs. II. Experimental evidence for acceptance of the "well-stirred" model over the "parallel tube" model using lidocaine in the perfused rat liver in situ preparation. *J. Pharmacokinetic. Biopharm.* 5: 655, 1977.
- Riedel, G. L., Schelle, J. L., Shepherd, A. P., and Ward, W. F. Effects of hematocrit on oxygenation of the isolated perfused rat liver. *Am. J. Physiol.* 245: G769, 1983.
- Hardison, W. G. M., and Wood, C. A. Importance of bicarbonate in bile salt independent fraction of bile flow. *Am. J. Physiol.* 235: E158, 1978.
- Mischinger, H. J., Walsh, T. R., Liu, T., Rao, P. N., Rubin, R., Nakamura, K., Todd, S., and Starzl, T. E. An improved technique for isolated perfusion of rat livers and an evaluation of perfusates. *J. Surg. Res.* 53: 158, 1992.
- Schmucker, D. L., and Curtis, J. C. A correlated study of the fine structure and physiology of the perfused rat liver. *Lab. Invest.* 30: 201, 1974.
- Curran, R. T., Gores, G. T., Thurman, R. G., and Lemasters, J. J. Protection by acidotic pH against anoxic cell killing in perfused rat liver: Evidence for a pH paradox. *FASEB J.* 5: 207, 1991.
- Hems, R., Ross, B. D., Berry, M. N., and Krebs, H. A. Gluconeogenesis in the perfused rat liver. *Biochem. J.* 101: 284, 1966.
- Thurman, R. G., Apel, E. D., and Lemasters, J. J. Protective effect of nitrendipine against hypoxic injury in perfused livers from ethanol-treated rats. *J. Cardiovasc. Pharm.* 12(Suppl. 4): S113, 1988.
- Jaeschke, H., Smith, C. V., and Mitchell, J. R. Reactive oxygen species during ischaemic-reflow injury in isolated perfused rat liver. *J. Clin. Invest.* 81: 1240, 1988.
- Rosini, S., Benetti, D., and Kvetina, J. The functional capacity of the isolated perfused rat liver in relation to the colloidal-osmotic composition of the perfusion medium. *Farm. Pract.* 31: 625, 1976.
- Exton, J. H., and Park, C. R. Control of gluconeogenesis in liver. I. General features of gluconeogenesis in the perfused livers of rats. *J. Biol. Chem.* 242: 2622, 1967.
- Huet, P. M., Kassissia, I., and Semret, M. Haemodynamics in perfused rat liver. In F. Ballet and R. Thurman (Eds.), *Perfused Liver: Clinical and Basic Applications*. London: Libbey, 1991. Pp. 33-42.
- Lemasters, J. J., and Thurman, R. G. Hypoxic, ischaemic and preservation-induced injury in the liver. In F. Ballet and R. Thurman (Eds.), *Perfused Liver: Clinical and Basic Applications*. London: Libbey, 1991. Pp. 97-120.

22. Hanks, J. B., Meyers, W. C., Wellman, C. L., Hill, R. C., and Jones, R. S. The effect of cell-free and erythrocyte-containing perfusion in rat livers. *J. Surg. Res.* **29**: 149, 1980.
23. Le Couteur, D. G., Rivory, L. P., Roberts, M. S., and Pond, S. M. Aging and the response of the isolated perfused rat liver to vasoactive drugs. *Biochem. Pharmacol.* **43**: 913, 1992.
24. Pang, K. S., Lee, W. F., Cherry, W. F., Yuen, V., Accaputo, J., Fayz, S., Schwab, A. J., and Goresky, C. A. Effects of perfusate flow rate on measured blood volume, disse space, intracellular water space, and drug extraction in the perfused rat liver preparation: Characterization by the multiple indicator dilution technique. *J. Pharmacokinet. Biopharm.* **16**: 595, 1988.
25. Greenway, C. V., and Lautt, W. W. Hepatic circulation. In S. G. Schultz (Ed.), *Handbook of Physiology: The Gastrointestinal System*. Bethesda, MD: Am. Physiol. Soc., 1989. Sect. 6, Vol. 2, Chap. 41, pp. 1519-1564.
26. Keiding, S., Vilstrup, H., and Hansen, L. Importance of flow and haematocrit for metabolic function of perfused rat liver. *Scand. J. Clin. Lab. Invest.* **40**: 355, 1980.