Reprod Nutr Dev (1997) 37, 29-40

Original article

Perfusion of trout liver in situ. Description and validation of the technique

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(Received 7 March 1996; accepted 12 September 1996)

Summary — The aim of this study was to develop a method for perfusing isolated trout livers that would make it possible to study hepatic metabolism in the whole organ. Rainbow trout (*Oncorhynchus mykiss* Walbaum) subjected to different fasting periods of 24, 48 or 96 h were used in all the experiments. A non-recirculating system was applied at a flow rate of 0.2 mL/min. The perfusion medium was a specific saline solution for salmonids oxygenated by a multibulb glass oxygenator. Viability assays included the measurement of oxygen consumption, lactate dehydrogenase activity and liver metabolic capacity. In addition, a histological study was carried out. Our results showed that the metabolic capacity of the liver survived throughout the perfusion process and that the functioning of this organ changed depending on the length of the fasting period to which the animal had been submitted. The method described here was shown to be suitable for studying the intermediate metabolism of fish.

rainbow trout / method / liver perfusion / fish / perfusion medium

Résumé — Perfusion de foie de truites in situ : description et validation de la technique. L'objectif de cette étude était de développer une méthode pour perfuser des foies isolés de truites, ce qui permettrait d'étudier le métabolisme hépatique dans l'organe entier in vivo. Les truites arc-en-ciel (*Oncorhynchus mykiss* Walbaum) ont été soumises à différentes périodes de jeûne de 24, 48 ou 96 heures dans toutes les expériences. Un système non-recirculant a été appliqué avec une vitesse de flux de 0,2 mL/min. La perfusion a été réalisée avec une solution saline, spécifique pour salmonidés, oxygénée pendant l'expérience par un oxygénateur en verre multibulles. La validation des essais a été basée sur la consommation d'oxygène, l'activité enzymatique de la lactate déshydrogénase et la capacité métabolique hépatique. De plus,

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une étude histologique a été réalisée. Nos résultats indiquent que la capacité métabolique du foie se maintient au long de l'opération de perfusion et que le fonctionnement de cet organe change en fonction de la durée du jeûne à laquelle ont été soumis les animaux. La méthode décrite paraît convenir pour l'étude du métabolisme intermédiaire chez les poissons.

truite arc-en-ciel / méthode / perfusion du foie / poisson / milieu de perfusion

INTRODUCTION

Liver perfusion has been widely applied in mammalian studies. In early assays, only the livers of larger animals were perfused, mainly dogs, because the surgical and analytical chemical techniques available were very limited. These techniques were gradually improved and began to be used with smaller animals, particularly rats (Corey and Britton, 1941; Trowell, 1942). Miller et al (1951) improved the method sufficiently for the results to be considered scientifically reliable. Currently this technique is even applied to small animals such as mice (Styne and Vierling, 1986).

The first studies using liver perfusion in fish date from the end of the 1960s. Pequin and Sefarty (1969) studied ammonia detoxication in perfused carp livers. Later, Hayashi and Ooshiro (1975) studied metabolism regulation in isolated eel livers.

Liver isolation offers many advantages. Methods using isolated tissue perfusion are useful in order to examine metabolic regulations at the tissue level, because the organ as a whole functions differently from isolated cells. It is known from microdissection studies of the liver that a number of enzymes have an unequal distribution along the hepatic sinusoid (for review, see Jungermann and Katz, 1982).

Liver perfusion makes it possible to study the exact metabolic use of many substrates by determining their concentration when they enter and leave the liver and thus allows the study of short- to medium-term effects of certain substances (endotoxins, medicaments, etc) on the liver. In addition, hormonal and nervous effects are avoided, as is the influence of metabolites from peripheral tissues, which could mask or interfere with the objectives under study (Rasenack et al, 1980; Schirmer et al, 1983; Quistorff and Grunnet, 1985).

The principal aim of this study was to develop a method for perfusing isolated trout livers, which would permit the study of hepatic metabolism in the whole organ and mimic the in vivo conditions as closely as possible.

MATERIALS AND METHODS

Animals

Forty-five rainbow trout (*Oncorhynchus mykiss* Walbaum) weighing 500 g were used in the perfusion experiments. Thirty of these were divided into three groups of ten animals, and each group was subjected to a fasting period of 24, 48 or 96 h. The remaining 15 trout were used as control subjects. These were divided into three groups of five animals each, with each group being subjected to the same fasting periods (24, 48 and 96 h) as the experimental animals. After anaesthesia, their livers were removed for posterior analysis.

Operative technique

Each trout was individually transferred to a small basin and anaesthetized by a mixture of trichloromethyl propanol and ethanol (3:1 by vol) added to the water (0.7 mL/L) for 5 min. Each animal was inverted on an operating board and the abdominal cavity opened by midline longitudinal and transversal incisions, with care being

taken to avoid transection of the major abdominal wall vessels. The animal was then anticoagulated through the dorsal aorta with an injection of 0.2 mL of a heparin solution (300 units/mL saline solution). Cannulation of the hepatic portal vein and the hepatic vein, both involved in liver perfusion, was then carried out (fig 1).

Hepatic portal vein cannulation

Two ligatures were placed around the hepatic portal vein approximately 2–6 mm from the point where it bifurcates towards the hepatic lobes. Both ligatures fixed the cannula within the hepatic portal vein. Other ligatures were placed on the collateral tributaries to the hepatic portal vein and another on the far side of the liver in order to block the circulation past the insertion point of the cannula. Next, an Abbocath-T n° 20G cannula (Abbot Ireland Ltd, Sligo, Republic of Ireland) was inserted into the vein and the ligatures were tied with a double knot. Preperfusion was immediately initiated with a well oxygenated saline solution in order to clean the liver of blood, the prevent blood clots in the hepatic sinusoids and to ensure that oxygen reached all the hepatocytes.

Hepatic vein cannulation

The longitudinal incision was lengthened to reach the anterior zone of the visceral cavity and another transversal incision was made as far as the operculum in order to remove the muscle wall, thus exposing the whole liver. Two ligatures were placed, one on the hepatic vein and the other on the coeliac artery, the first to fix the cannula and the second to block circulation and the outflow of the perfusion liquid. Finally, an Abbocath-T n° 18G cannula (Abbot Ireland Ltd) was inserted into the vein and the ligatures were tightened.

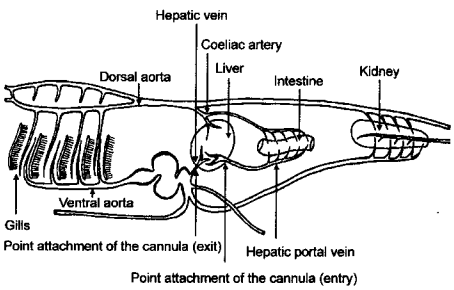

Fig 1. Diagram showing the point of attachment of the cannula to the hepatic portal vein and the hepatic vein.

Perfusion system

The system used in our study was based on the design of Miller et al (1951) and Schimassek (1963), later described by Hems et al (1966). However, the substantial anatomical and physiological differences between rat and trout meant that certain modifications were necessary, particularly with regard to the circuit type, perfusion medium composition inflow speed, form of oxygenation and temperature. The experiments

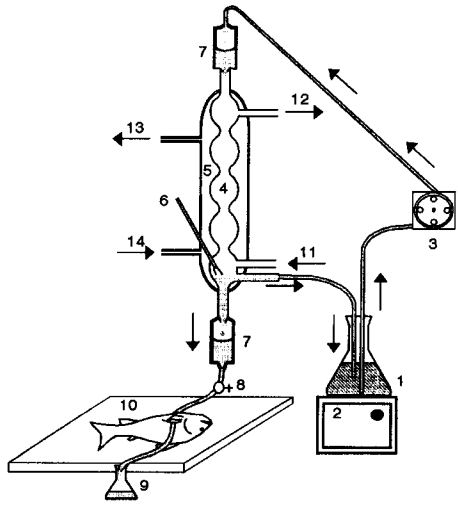

Fig 2. Diagram showing the isolated perfused trout liver model: (1) collecting vessel; (2) magnetic stirrer; (3) peristaltic pump; (4) multi-bulb glass oxygenator; (5) double jacket; (6) thermometer; (7) filter; (8) inflow to liver; (9) outflow from liver; (10) platform for animal; (11) gas inlet; (12) gas outlet; (13) water outlet; and (14) water inlet.

were performed in a room with a constant temperature and humidity.

A diagram of the system is shown in figure 2. The medium in the collecting vessel (1) was stirred with a magnetic stirrer (2), which continuously homogenized the perfusion medium. The perfusion medium was pumped from the collecting vessel by a MicroPerpex LKB 2132 peristaltic pump (3) to the top of the multi-bulb oxygenator (4). The perfusion medium temperature was almost constant (14 ± 1 °C) since the water came from a thermostatic bath and passed through a double jacket (5) enveloping the oxygenator during perfusion. The temperature was controlled by a contact thermometer (6). The system's components were connected by transparent vinyl tubing except for the piece directing the overflow from the reservoir to the collecting vessel, which was of highly flexible silicone tubing. A non-recirculating system was used with a flow rate of 0.2 mL/min (12 mL/h). The medium was oxygenated with a humidified mix of O_2:CO_2 (95:5) supplied by a multi-bulb glass oxygenator in which the perfusate was oxygenated using large fluid–gas interfaces. The gas was previously saturated with water by bubbling it through a wash bottle (Miller et al, 1951; Schimassek, 1963; Hems et al, 1966).

The perfusion medium used in our experiments was a specific saline solution for salmonids (Holmes and Stott, 1960), pH 7, containing 250 mM NaCl, 9.6 mM KCl, 33.7 mM Na_2HPO_4, 6.6 mM NaH_2PO_4, 4.5 mM $CaCl_2$, 7.2 mM CO_3HNa and gentamicin (100 mg/L; 64 400 IU/L). It was kept refrigerated for a maximum of 10 days before use. Before the liver perfusion was performed, the medium was subjected to an equilibrating period by being passed through the perfusion circuit for 20 min, thus reaching the appropriate oxygen, pH and temperature levels.

Sample collecting

After the liver had been connected to the perfusion circuit, the organ was stabilized for 10 min and the perfused medium collected during this time discarded. Next, samples were collected by taking the whole volume of eluent every 15 min for 2 h. These samples were centrifuged at 1 000 g for 15 min and frozen at −18 °C.

The oxygen concentration and pH of the perfusion medium were measured by taking several samples with a syringe intermittently throughout the experiment (15, 45, 75, 105 min) from the lower part of the reservoir (immediately before entering the liver) and as the medium left the organ. In this way the oxygen consumption by the liver tissue could be calculated.

Difficulties in cannulating the bile duct prevented the bile flow rate from being measured. In both the control and perfused animals (once the perfusion was completed), the liver was dissected and weighed. Part of the liver (0.5 g) was homogenized in order to assess the lactate dehydrogenase (LDH) activity (Gollan et al, 1981; Guaitani et al, 1983); another part was used for histological examination, and the rest was desiccated in an oven (105 ± 1 °C) to assess the dry weight. Homogenization was achieved with ten volumes of 58 mM potassium phosphate buffer, pH 7.5, in a Polytron homogenizer for 20–30 sec at 4 °C.

Analytical methods

The perfusion medium and eluent were assessed for glucose, LDH activity, partial oxygen pressure and pH as detailed here.

Glucose

By enzymatic test (Böhringer-Mannheim, n° 676551), based on the glucose-oxidase peroxidase method.

LDH activity

According to the method described by Bergmeyer and Bernt (1974), recording NADH oxidation spectrophotometrically.

Partial oxygen pressure and pH

Perfusion medium samples collected before and after passing through the liver throughout the whole perfusion process were immediately measured in a gasometer (ABL2 Acid-Base, Radiometer Denmark).

Histological examination

Cells of perfused and non-perfused trout livers starved for 24 h were observed with toluidine blue staining. Mucopolysaccharides of non-perfused trout livers starved for 24 and 96 h were observed using the periodic acid-Schiff method (PAS) (McManus, 1948).

Statistical analysis

The data are expressed as means ± SEM. Significance tests were carried out using the Student's *t*-test, multiple variance analysis and a non-parametric Kruskal-Wallis test. Probability of less than 0.05 were considered significant.

RESULTS

Figure 3 shows the glucose production (µmol/min•g dry liver) every 15 min for a total time period of 120 min in isolated trout livers submitted to different fasting periods of 24, 48 or 96 h after perfusion with a specific saline solution for salmonids. Glucose production was highest during the first period (15 min). It then gradually decreased until the fourth period (60 min), to become more or less stable until the end of perfusion (fig 3). The hyperglycaemia observed during the first part of the perfusion was probably a result of stress (a transfer, an anaesthetic, an heparin injection, etc), as has previously been shown

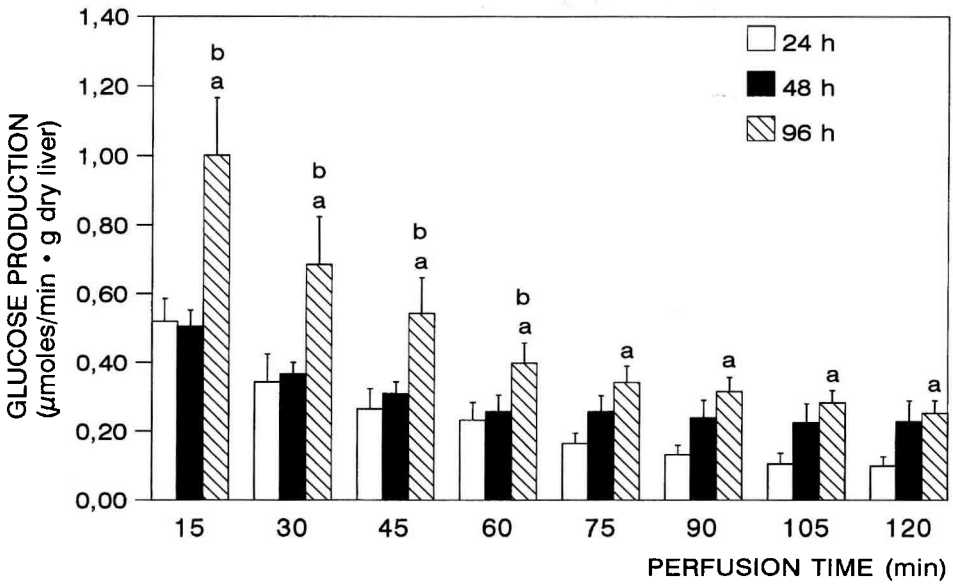

Fig 3. Glucose production in isolated trout livers submitted to different fasting periods: 24, 48 or 96 h during perfusion with saline solution every 15 min for a total of 120 min. The data are expressed as means (n = 10) and SEM is shown by the vertical bars. [a] Significant differences between 24 and 96 h fasting; [b] significant differences between 48 and 96 h fasting, $P < 0.05$ (Student's t-test).

(Morales et al, 1990). When the effect of the fasting period was studied, there was a statistically significant increase ($P < 0.05$) in glucose production throughout the perfusion in animals starved for 96 h when compared with animals starved for 24 and 48 h.

There was no difference in LDH activity in the liver homogenate between the perfused and non-perfused liver (191 ± 51 and 200 ± 42 µmol/min•g fresh liver, respectively) in trout starved for 24 h. The LDH activity of the eluent was very low (< 0.02 x 10^{-3} µmol/min•g fresh liver), about 0.006% of the LDH activity in the liver during the first 15 min of perfusion. No LDH activity was detected in the eluent after this time.

The mean value of oxygen consumption by isolated trout livers starved for 24 h, during the 120 min of perfusion, is shown in figure 4. These values represent about 0.12 µmol/min•g liver.

Perfused (fig 5A) and non-perfused (fig 5B) trout liver sections starved for 24 h. Both showed a well preserved cell structure. Nevertheless, the sinusoids appeared to be dilated in the perfused liver, which is characteristic of the perfusion process.

The PAS (+) content in non-perfused trout liver sections starved for 24 h (fig 6A) and 96 h (fig 6B) shows a marked depletion of the hepatic glycogen reserves in the fish livers starved for 96 h compared with those starved for 24 h.

DISCUSSION

Methodological conditions

The successful outcome of the operation depends on several methodological factors: the surgical operation itself, the circuit type, composition, pH and temperature of the perfusion medium, inflow rate and oxygenation system. In addition, it is necessary to validate liver perfusion in fish by a series of tests in order to assess isolated liver viability during the perfusion process. The most frequently

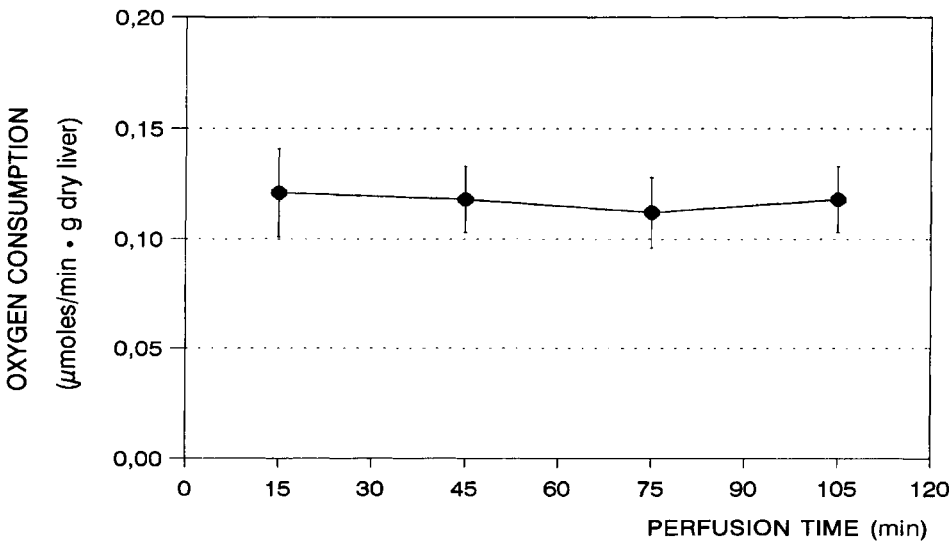

Fig 4. Oxygen consumption by isolated trout livers during 120 min of perfusion. The data are expressed as means ($n = 10$) and SEM is shown by the vertical bars.

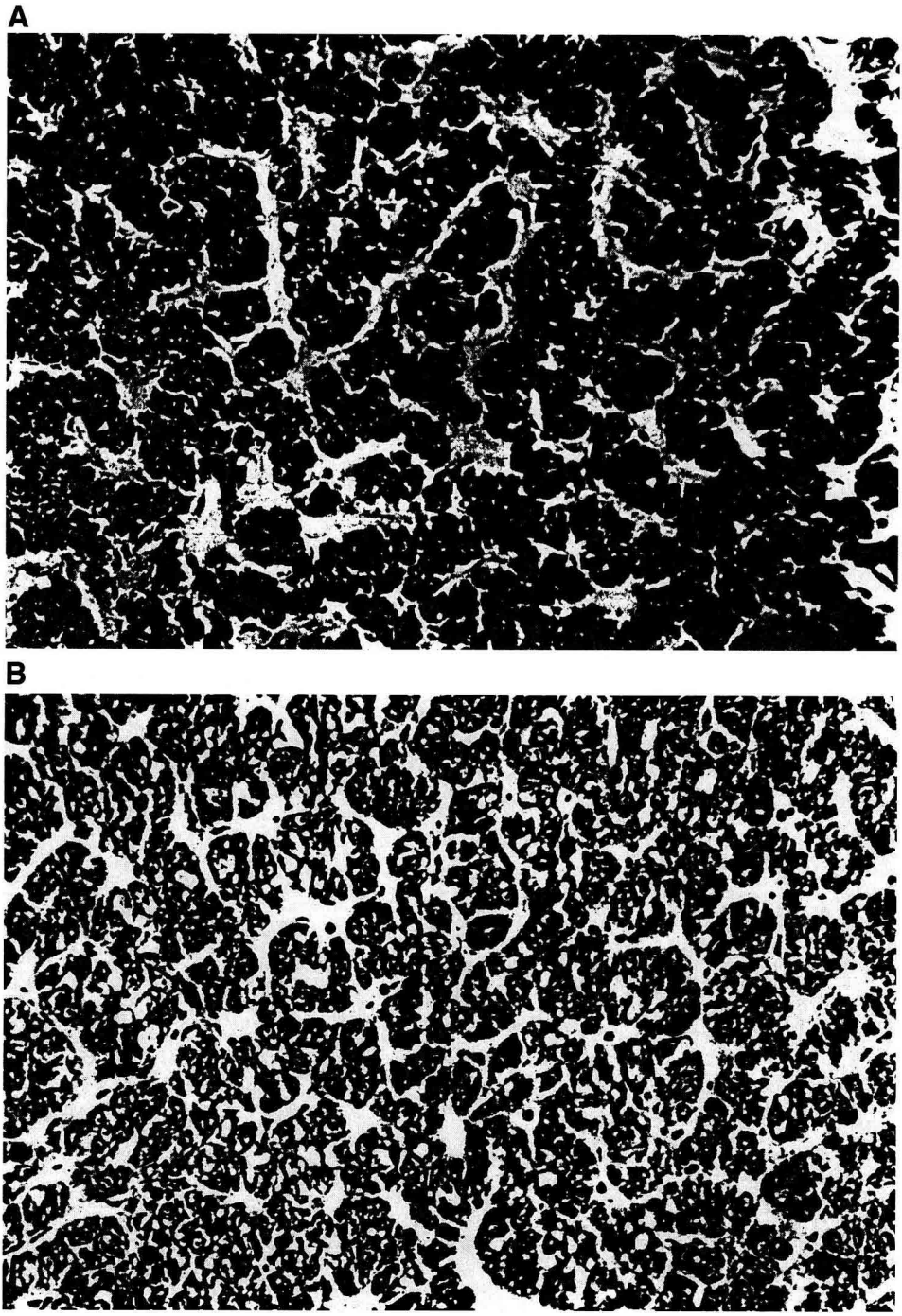

Fig 5. Light micrograph of toluidine blue stained sections of perfused (**A**) and non-perfused (**B**) trout livers starved for 24 h. Typical structure was maintained (x 420).

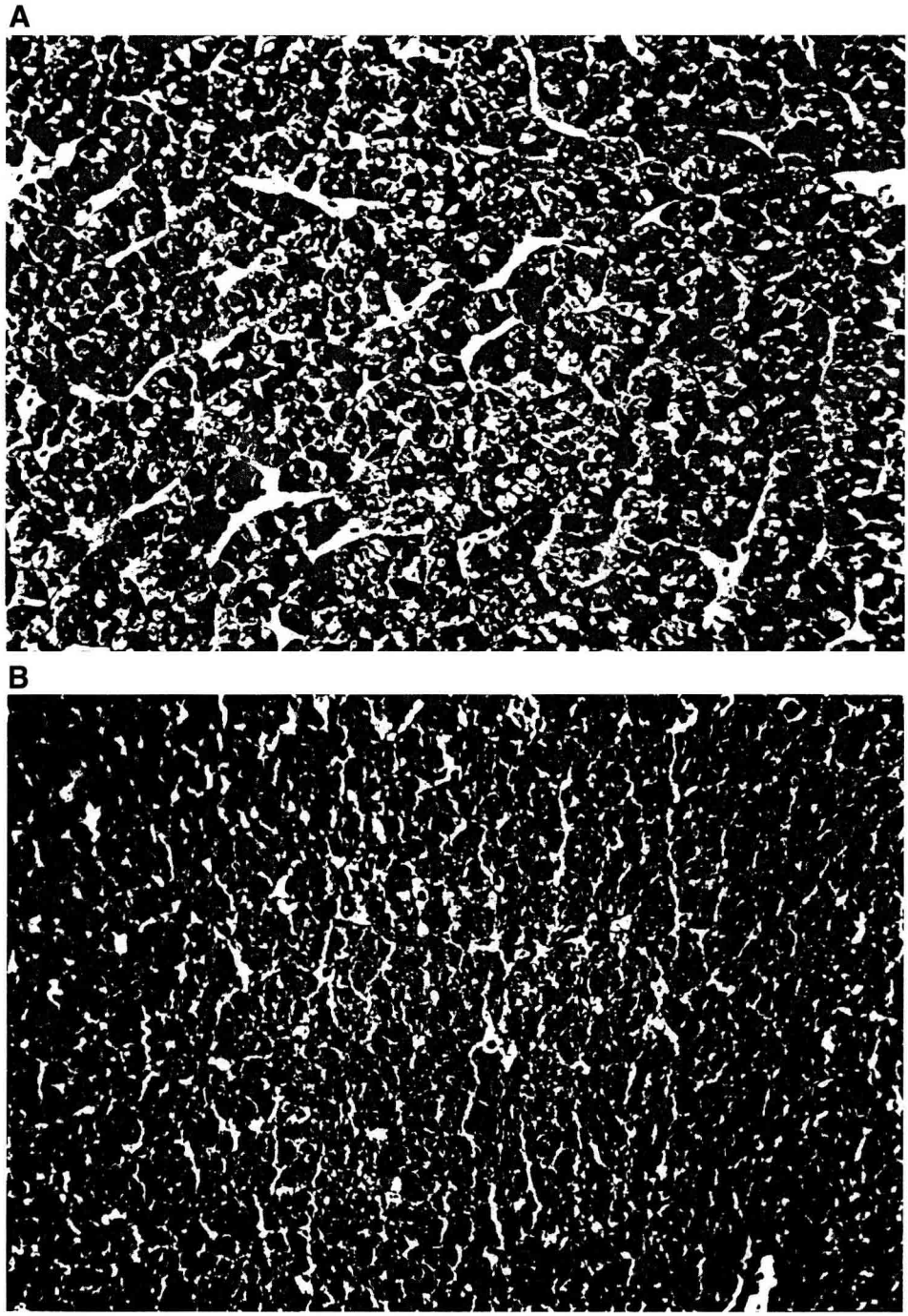

Fig 6. Light micrograph of periodic acid-Schiff (+) stained sections of non-perfused trout livers starved for 24 h (**A**) and 96 h (**B**). A high glycogen reserve can be observed in A, while B shows a depletion (x 420).

used validation methods involve measuring oxygen consumption, bile flow, hepatic enzyme activity and histological examination of the liver.

When both veins are cannulated, there is an interruption of the blood circulation and the liver is deprivated of the oxygen that is necessary for its survival and normal functioning. To minimize the length of the anoxic period, the operation must be carried out as quickly as possible.

Furthermore, the operation is more complicated in fish because after anaesthesia the animal is removed from the water. It is therefore very important that after cannulation of the hepatic portal vein and before hepatic vein cannulation, the organ should be connected to a preperfusion circuit containing a very well oxygenated medium; even so, the anoxic period should not exceed 5 min if irreversible liver damage is to be avoided.

A variety of perfusion media have been used for this technique. Some researchers (Miller et al, 1951; Brauer et al, 1954) have used blood so that the experimental conditions would resemble in vivo conditions as closely as possible. However, this involves large volumes of fresh blood, which must be pretreated before perfusion in order to avoid metabolic interactions and clot formation. For this reason a semisynthetic medium, first used by Schimassek (1963), has frequently been employed.

We used a saline solution isosmotic with the internal medium of the animal (Holmes and Stott, 1960). This is a simple medium which is inexpensive and easy to make because it does not contain albumin or erythrocytes. Based on the results of the different viability assays, we verified that this perfusion medium, consisting only of a very well oxygenated saline solution, was adequate for this kind of research, and our results agree with those of other authors (Fröhlich et al, 1973; Oshino et al, 1973; Oshino and Chance, 1975).

Since even slight changes in the pH and temperature of the perfusion medium significantly affect protein and carbohydrate metabolism in the perfused liver (Miller and Griffin, 1973), these parameters must be strictly monitored during the perfusion process. The usual pH and temperature values used with this kind of technique are those of the animal in in vivo conditions (Keiding et al, 1980; Reed et al, 1982; Styne and Vierling, 1986).

In our experiments, the perfusion medium had a constant temperature and pH throughout the 2 h perfusion (14 °C and 7.0, respectively).

Two different oxygenation systems have traditionally been used for the perfusion medium: a multi-bulb glass oxygenator (Miller et al, 1951; Schimassek, 1963; Hems et al, 1966) and a membrane oxygenator (Scholz et al, 1973; Gollan et al, 1981). Even though both methods have been described in the literature as providing sufficient amounts of oxygen to cover the liver requirements, we chose to use the former because under our experimental conditions, the amount of oxygen in the perfusion medium was significantly higher ($P < 0.05$) when the multibulb glass oxygenator was used. Moreover, the amount of oxygen consumed by the liver was significantly higher ($P < 0.001$) for this system.

The optimum perfusion flow for each species must be sufficiently high to ensure an adequate supply of oxygen to all parts of the liver but not exceed a limit, as this would result in tissue damage. In mammal liver perfusion studies (rat, mouse, rabbit), the optimum perfusion flow values have been set between 0.5 and 2 mL/min (Reichen and Paumgartner, 1976; Hartmann et al, 1982; Guaitani et al, 1983; Styne and Vierling, 1986), values which are similar to hepatic blood flow rates in vivo. Reed et al (1982) however, showed that it was necessary to use lower perfusion pressures during fish liver perfusion because portal pressure values in fish are lower than those in mammals. Furthermore, the perfusion process has to be initiated with a very low pressure compared to that used with the rat, where the initial hepatic

resistance is higher. After different validation assays we chose a perfusion flow of 0.2 mL/min (12 mL/h) for the trout liver perfusion.

Validation assays

To verify the validity of this method, the liver was submitted to different viability assays to determine whether the organ remained in a satisfactory condition during and after the perfusion process. Among the assays were the measurement of oxygen consumption, LDH activity and glucose production in the isolated liver. In addition, the perfused liver was subjected to histological study (Diaz and Connes, 1988; Gonzalez et al, 1993; Rocha et al, 1995).

Oxygen consumption is one of the most reliable criteria for assessing tissue damage during perfusion, since it is possible to ascertain whether the process takes place homogeneously in the whole liver or whether there are some regions where oxygenation is insufficient.

In our study, liver oxygen consumption was almost constant at around 0.12 μmol/min•g throughout the perfusion (fig 4), implying that metabolic activity continued during the perfusion process and that the liver was in a similar condition at the end of the experimental period as at the beginning. These oxygen consumption values were lower than those for mammals given in the literature (Tavoloni et al, 1978; Gollan et al, 1981), but were similar to other values in fish described by different authors (Reed et al, 1982; Blanco, 1994).

Another criterion frequently used for validating this kind of technique is LDH activity (Rasenack et al, 1980; Gollan et al, 1981; Guaitani et al, 1983). In our experiments, almost no enzyme leaked from the cells into the perfusion medium during perfusion. Only in some samples during the first 15 min, was a very low LDH activity detected with respect to unperfused liver. This was probably due to the slight traumatism resulting from the initial intake of the perfusion medium into liver. LDH activity values in the perfused liver were similar to those in the non-perfused liver, suggesting that this activity was unchanged by the perfusion process.

The appearance of the liver, particularly with regard to colour homogeneity, is another possible sign which can indicate the success of the technique. A uniform colour is more important than colour intensity, because the latter depends on the composition of the perfusion medium, while uneven coloration is a sign of uneven perfusion.

A few minutes after the beginning of perfusion, we observed that the liver appeared homogeneously discoloured, indicating that the medium had reached all the regions of the organ.

Microscopic examination of the structure and ultrastructure of the perfused liver is another reliable test of the viability of the perfused tissue. The integrity of the perfused liver can be upset by different factors (excessively high inflow, cell anoxia, inadequate temperatures, etc), as shown in several studies that have compared non-perfused and perfused liver sections by means of optic and electronic microscopy (Sugano et al, 1978; Nashat et al, 1985; Styne and Vierling, 1986).

Microscopic observation of the perfused and non-perfused liver sections did not indicate any structural modifications of the liver during perfusion. No changes in cell integrity after perfusion and no vacuolization or necrosis were observed (fig 5).

When the liver was perfused with a specific saline solution for salmonids, we observed that, independently of the fasting period, glucose production was at a maximum during the first period of the experiment, probably due to the effect of stress (Morales et al, 1990). It then decreased progressively until the fourth period, to remain more or less stable until the end of perfusion (fig 3). It also

appears that the changes in glucose levels may be, at least in part, a consequence of the state of the animal at the moment it was subjected to perfusion; that is, a consequence of the hormonal state prior to liver isolation. This would have an effect on the hepatic enzymes, provoking activation or inhibition, and this was then measured indirectly by means of different metabolites.

When the influence of fasting on glucose production or liberation was studied, a change in the metabolic capacity of the liver was observed. For the fish that had undergone 96 h of fasting, the glucose production was significantly higher ($P < 0.05$) than for those undergoing 24 and 48 h of fasting for every 15 min interval period. There were no differences between the 24 and 48 h fasted fish. This may have been because only gluconeogenesis is activated during short fasting periods (24 and 48 h), which supposes that hepatic proteolysis is also taking place. Under the more drastic conditions (96 h of fasting), regardless of the higher gluconeogenesis levels, a glycogen hydrolysis process would also be activated.

In summary, our results showed that the liver survived throughout the perfusion process and that the functioning of this organ changed depending on the fasting period to which the animal was previously submitted.

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