

## Examination of Intrahepatic Flow Distribution by Vital Staining With Rhodamine 6G in Isolated Perfused Rat Liver

Yasusuke Masuda and Tamami Oguma

*Division of Toxicology, Niigata College of Pharmacy, 5-13-2, Kamishin'ei-cho, Niigata 950-2081, Japan*

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**ABSTRACT**—We previously reported that intrahepatic flow disturbance can be detected by vital staining of the liver with a fluorescent dye (Masuda et al., *Biochem Pharmacol*, **53**, 1779–1787 (1997)). To evaluate further use of this method, a detailed study was performed. The isolated perfused rat liver was vitally stained with rhodamine 6G (R-6G) and perfusion-fixed, and cross and horizontal sections were examined by fluorescence microscopy. In the control liver, R-6G staining was localized to periportal hepatocytes and was distributed evenly throughout the liver, indicating a homogeneous perfusion. Finer examination of the thick sections and reconstruction of a three-dimensional flow pattern revealed intricate vascular networks together with the sinusoids in different portions of the liver. In a flow-redistribution model, i.e., under hepatic nerve stimulation, the extensive flow redistribution to the deeper portion of the liver was found to occur via short branches sprouted from large portal veins, with minimal perfusion of the liver margin. Thus, visualization of hepatic microvasculature enables anatomical analysis of flow disturbance. The method is indirect but simple and may help detect intrahepatic flow disturbance that could be evoked by various factors.

**Keywords:** Intrahepatic flow disturbance, Hepatic vascular network, Rhodamine 6G, Isolated perfused rat liver, Hepatic nerve stimulation

The liver receives blood from the portal vein (PV) and the hepatic artery, especially from the former. These inlet vessels repeatedly ramify to fine branches and continue into the sinusoids, and the blood finally drains into the hepatic vein (HV) (1). Hepatic microcirculation is of primary importance for metabolic functions of the liver (2). Therefore, it is important to develop a method for detecting intrahepatic flow disturbance that could be produced by various factors.

Various methods have been used to detect intrahepatic flow, including radioangiography (3, 4), trypan blue staining (5), microsphere distribution (6), heat conduction (7), ultrasonic volume-flowmetry (8), laser Doppler flowmetry (9) and the multiple-indicator dilution method (10). However, these methods are macroscopic or not visual. Microscopy of the trans-illuminated liver edge (11) or the epi-illuminated liver surface after vital fluorescent labeling (12) is a powerful method for real-time observation of sinusoidal flow. However, deeper portions of the liver cannot be examined by these techniques. As an alternative method, microscopy of cryo-fixed liver sections following injection of fluorochrome-bound plasma proteins has been reported (13).

In our previous study with isolated perfused rat livers

(IPRL) (14), the liver was vitally stained with the fluorescent probe acridine orange (AO), fixed in formalin, sectioned and then examined under a fluorescence microscope. This method, which is based on the selective uptake of some lipophilic fluorescent probes by periportal hepatocytes (15), allows visualization of real flow distribution throughout the liver mass, although it is static and indirect. By combination of this method with a multiple-indicator dilution technique and distribution of fluorescent beads, some vasoconstrictors were found to cause a redistribution of intrahepatic flow to the central portion of the liver (14). Hitherto, however, microscopic observations by this fluorescence labeling method were only made with thin cross sections, and close examination has not been accomplished yet.

In the present study, to evaluate further use of the method, we first examined in detail sinusoidal flow distribution together with intrahepatic vascular networks in control IPRL, by using liver sections cut in various thicknesses and different directions and by reconstructing a three-dimensional flow model. Then, the flow-redistribution during hepatic nerve stimulation which has been shown by many investigators by means of other methods (4–9) was re-examined. For this, rhodamine 6G (R-6G) was used as a

fluorescent probe.

## MATERIALS AND METHODS

### *Animals*

Male, SPF-grade Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu) and housed in an air-conditioned animal room (temperature,  $23 \pm 1^\circ\text{C}$ , humidity, 50–60%) supplied with fresh clean air, with food and water given ad libitum. Well-nourished animals with a body weight of 220–230 g were used throughout the experiments. Animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, approved by The Japanese Pharmacological Society.

### *Liver perfusion system*

The liver was isolated under pentobarbital anesthesia and perfused essentially as described previously (14). To minimize possible uneven perfusion among the lobes during disturbed circulation, median and left lobes were isolated and perfused, while the other smaller lobes were tied and cut off. For this, the tip of the inlet cannula was advanced close to the bifurcation of the PV to the median and left lobes. The outlet cannula was placed through the right atria into the vena cava, with the tip advanced to the entry of HV. The bile duct was cut. The liver was suspended in the perfusion medium kept at  $37^\circ\text{C}$  and perfused in a non-recirculating, constant flow (20 ml/min) system, using a rotary pump. The perfusion medium was Krebs-Henseleit bicarbonate buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 25 mM  $\text{NaHCO}_3$  and 5.6 mM glucose, saturated with 95%  $\text{O}_2$ –5%  $\text{CO}_2$  at  $37^\circ\text{C}$ , pH 7.4) containing 2 mM sodium lactate and 0.2 mM sodium pyruvate. The point of the outlet cannula was set at the level of the liver hilum, and portal perfusion pressure (PP) was monitored using a pressure transducer (Saftikit, DSK-101; Kawasumi Laboratories, Inc., Tokyo). The position of the liver was adjusted to maximize the hepatic oxygen consumption using a  $\text{pO}_2$  monitor (Type  $\text{PO}_2$ -100; Inter Medical Co., Ltd., Nagoya). Experiments were started 30 min after portal cannulation. The post-fixed weight of perfused livers was about 6.3 g.

### *Vital staining with rhodamine 6G (R-6G) and microscopy*

In this study, we used the lipophilic fluorescent probe R-6G (Sigma Chemical Co., St. Louis, MO, USA) to stain periportal areas. R-6G was infused into the perfusion line at a concentration of  $1.25 \mu\text{M}$  for 1 min (25 nmol/liver). The effluent perfusate was collected for 4 min after starting R-6G infusion, if necessary. In some experiments, after R-6G staining, the liver perfusion system was switched to a retrograde direction, i.e., from outlet (hepatic venous) to

inlet (portal venous) direction, and AO (Chroma Gesellschaft Schmidt GMBH & Co., Köngen, Germany) (usually 50 nmol/liver) was infused to stain perivenous areas; this additive manipulation visualizes HV branches. Then, the liver was perfusion-fixed for 4 min with 2% glutaraldehyde solution containing 0.09 M sodium phosphate buffer (pH 7.4) and 1% saccharose (total, 430 mOsM). This rapid fixation was superior to the previous dip-in method (14) in preventing bleaching and diffusion of the dye.

Liver sections of appropriate thickness (50–500  $\mu\text{m}$ ) were prepared in cold 0.1 M phosphate buffer (pH 7.4) containing 10% glycerol using a microslicer (DYK-1000; Dosaka EM Co., Ltd., Kyoto). Two types of liver sections were made. Cross-sections were cut from three parts of the left lobe (L-1, hilar side; L-2, middle; and L-3, longitudinal edge) and from the middle part (M-2) of the median lobe. For horizontal sections, the left lobe was divided into the anterior and posterior parts, and the deeper portions where large vessels are located were cut.

The sections were mounted on slide glass and photographed under green (546 nm) or blue (405, 435 and 490 nm) excitation using a fluorescence microscope (BH2-RFCK; Olympus Optical Co., Ltd., Tokyo). R-6G showed red fluorescence on a dark background on excitation with weak green light (low magnification), but showed yellowish orange fluorescence on a red background under strong light (high magnification). It also fluoresced yellow on blue light illumination. AO fluoresced green on a dark background or light yellowish green on a green background, depending on the strength of blue light. AO fluorescence on green excitation was very weak.

### *Reconstruction of three-dimensional (3-D) structures*

The liver was vitally stained with 50 nmol of both R-6G (antegrade) and AO (retrograde). The concentration of R-6G was raised to increase photographic contrast under blue light excitation. More than 20 serial sections (50  $\mu\text{m}$ ) of the left lobe were cut, illuminated with blue light and photographed. Periportal and perivenous sinusoids were traced on transparent films with red and blue ink, respectively, and the films were overlapped at 50- $\mu\text{m}$  equivalence and photographed.

### *Hepatic nerve stimulation*

Platinum wire electrodes (diameter, 0.2 mm) were attached to the bifurcation of the extrahepatic PV to the median and left lobes, and the hepatic nerve plexus was stimulated at 20 V for 2 ms with a frequency of 2–50 Hz, using an electronic stimulator (DPS-05; Dia Medical System Co., Ltd., Tokyo). In the stimulated livers, R-6G was infused when the PP increased to the maximum level.

### Data presentation and statistical analysis

Representative pictures for each group are shown. Numerical values are represented as means  $\pm$  S.E.M. Statistical analysis for unpaired values was performed by the method of Bonferroni after one-way analysis of variance. For paired values, the paired *t*-test was used.  $P < 0.05$  was considered significant.

## RESULTS

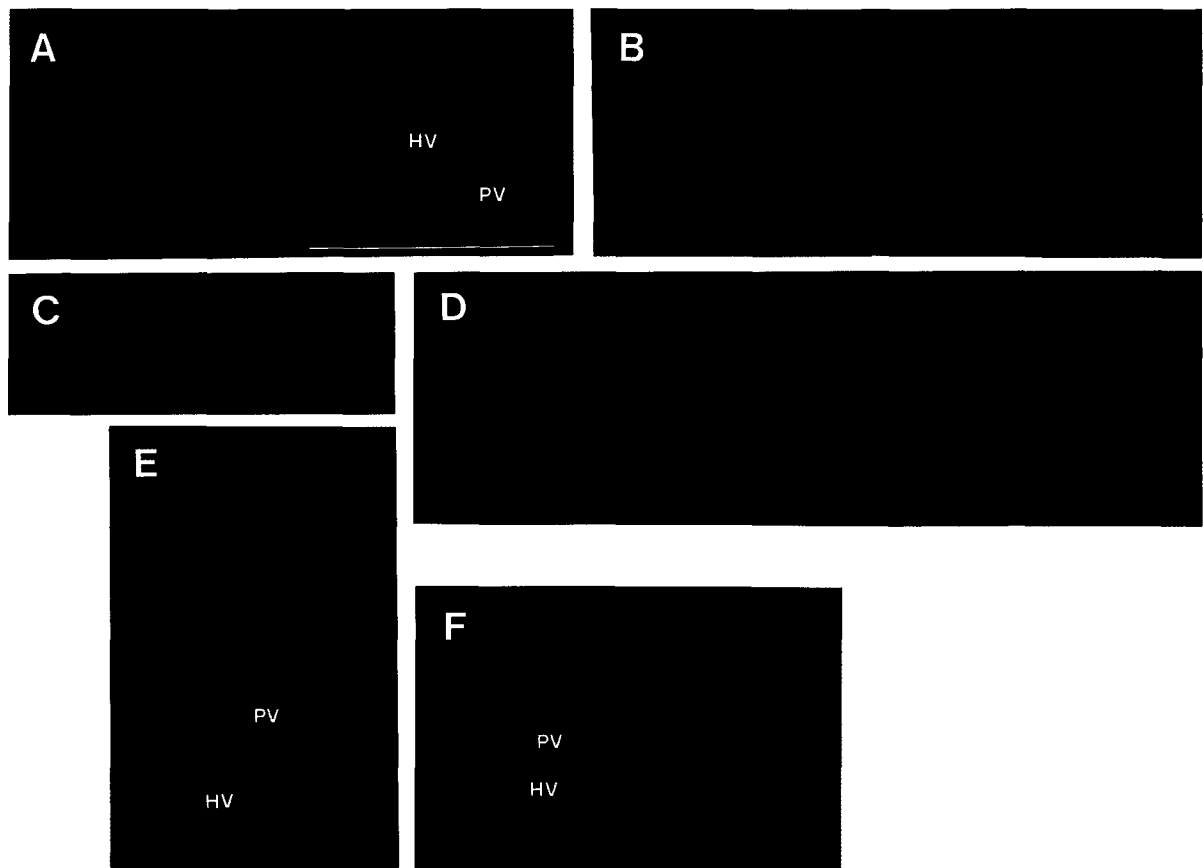
### R-6G staining pattern in the control IPRL

As shown in the low magnification pictures (Fig. 1), cross-sections of the left lobe (A–C, from hilar to longitudinal edge) and the median lobe (D, middle) and horizontal sections of the left lobe (E and F, anterior and posterior parts, respectively) all showed even distributions of bright areas of R-6G fluorescence, and thus the liver was homogeneously perfused.

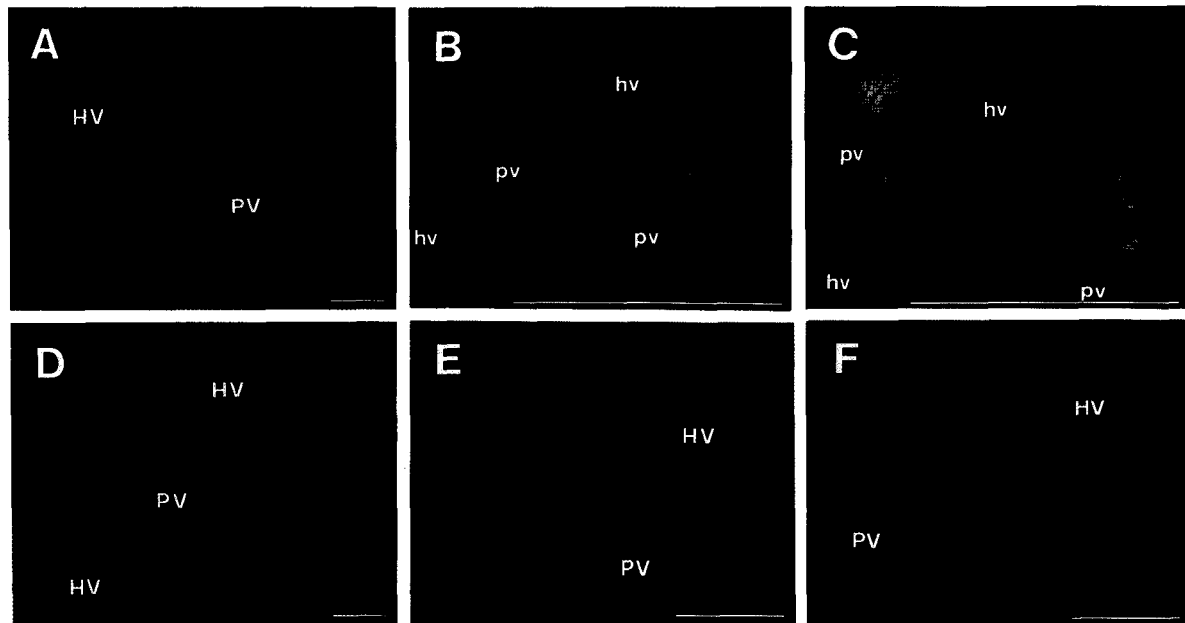
Selective staining of periportal hepatocytes was evident under higher magnification (Fig. 2). At the margin of the liver, the periportal staining around final PV branches spread toward the liver surface (A and B). In the inner portion, HV branches of various sizes passed through the stained areas (A and C). Horizontal sections revealed that large conducting PVs gave rise to many short vertical branches (D) and also a fine distribution of veins running close to the wall of large PVs (E and F, arrows and arrow-heads), with distinct staining around these branches. Thus, the hepatocytes located close to large PVs were well perfused.

### Dual staining with R-6G and AO

To clearly visualize perivenous sinusoids and the draining vessels, AO was infused in a retrograde manner after R-6G staining. As shown in Fig. 3 (A vs B, and E vs F), green light excitation allowed visualization mainly of the



**Fig. 1.** Fluorescence microscopy of post-fixed liver sections after vital staining with R-6G in the isolated perfused rat liver (IPRL) (low magnification). The IPRL was infused with 25 nmol/liver of R-6G, perfusion-fixed, and liver sections were prepared. A–D: Cross sections (50- $\mu$ m-thick) of L-1 (hilar side) (A), L-2 (middle) (B) and L-3 (longitudinal edge) (C) of the left lobe; and M-2 (middle) of the median lobe (D). E and F: Horizontal sections (50- $\mu$ m-thick) of anterior (E) and posterior (F) parts of the left lobe. PV: Large portal vein, HV: large hepatic vein. Representative photos from 5 and 3 livers in cross and horizontal sections, respectively, are shown. The bar indicates 1 cm. All cross sections and horizontal sections were evenly stained, indicating homogeneous perfusion throughout the liver.



**Fig. 2.** Fluorescence microscopy of post-fixed liver sections after vital staining with R-6G in IPRL (higher magnifications). A – C: Cross sections of L-1, 50- $\mu$ m-thick. D: Horizontal sections of the anterior part of the left lobe, 50  $\mu$ m. A – D are from the experiments shown in Fig. 1. E and F: Same portion as in D, 500  $\mu$ m, obtained from 3 additional experiments. Bars indicate 1 mm. Three features of the periportal staining are shown. At the liver margin, the staining spread from terminal portal venules (pv) toward the surface, interrupted by terminal hepatic venules (hv) (A and B). In the inner portion, periportal staining along longitudinal or cross-sections of the portal venules was interrupted by central veins of various sizes (A and C). Large portal veins (HV) give rise to many short branches, perpendicular to (arrows) or in parallel with (arrowheads) the portal vein wall (D – F), with strong staining around these areas.

periportal areas stained by R-6G (B and F), whereas blue excitation of the same sections visualized both portal and hepatic venous areas stained by R-6G and AO, respectively (A and E); although the discrimination of the R-6G (yellow) and AO (yellowish green) fluorescence is difficult in the picture, both were discernible under the microscope or on posi-films. The portal inflow into sinusoids was seen to be directed towards interdigitating HV branches (hv) of various sizes with an approximate distance of 400  $\mu$ m between portal and hepatic venous branches (A). Likewise, at the margin of the liver, a final PV branch gave flow into the upward as well as lateral sinusoids which naturally continue to nearby HV branches (C). Microscopy of the liver surface (D) also indicated a fountain-like flow at the surface, from a final PV branch to the surrounding final HV branches.

In the hilar portion of the liver (E and G, cross and horizontal sections, respectively), the smaller PV branches sprouted from larger PVs interdigitated with small HV branches draining into larger HVs. Large HVs had three types of inlet vessels as visualized by AO staining (H): main large branches that were not stained perivascularly (asterisk), smaller vertical branches that were abundant and stained perivascularly (arrows), and finer branches dis-

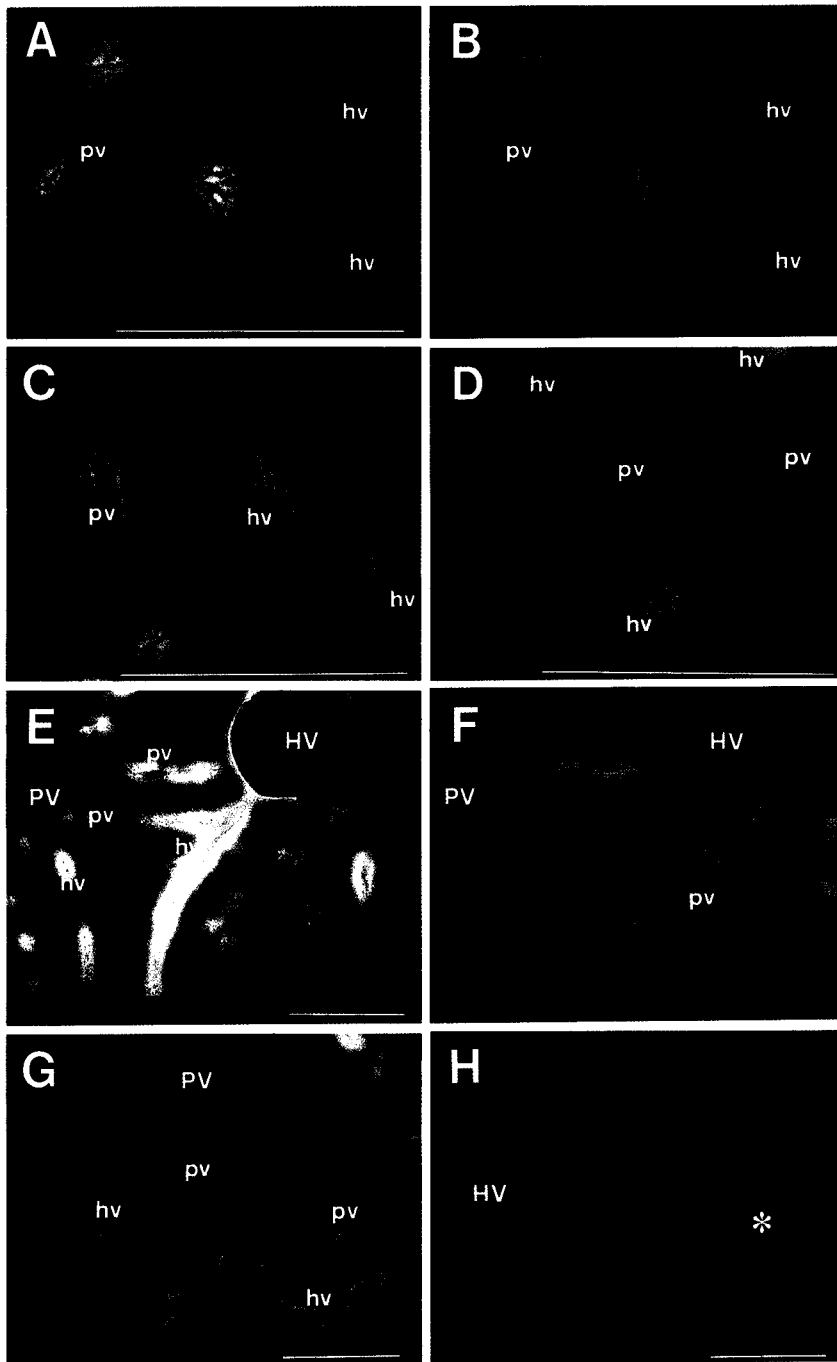
tributed on the wall of the HV trunk (arrowheads).

### 3-D flow models

To examine the steric features of hepatic microcirculation, 3-D flow models were reconstructed from the dual-stained liver samples (Fig. 4). At the surface (A and B), interdigitation of portal and hepatic venules and their connecting sinusoids was seen. Not only the final portal branches (arrowheads) but their pre-branching segments (arrows) appeared to provide flow to sinusoids. At the edge (C), a terminal PV branch burrowed towards the utmost edge to provide flow to the marginal sinusoids. At the hilar portion (D), particularly where large PV and HV run in close proximity, their short branches were interdigitated. Thus, intricate branching of PV provided flow to all regions of the liver.

### Flow disturbance during hepatic nerve stimulation

To validate our IPRL-perivascular stimulation system, the effects of electrical stimulation on PP were first examined. Stepwise increases of stimulation frequency (2 – 50 Hz, at 20 V, for 2 ms) produced a cumulative increase in PP, with a maximum response reached at 20 Hz (Fig. 5A), which was suppressed in a concentration-dependent man-



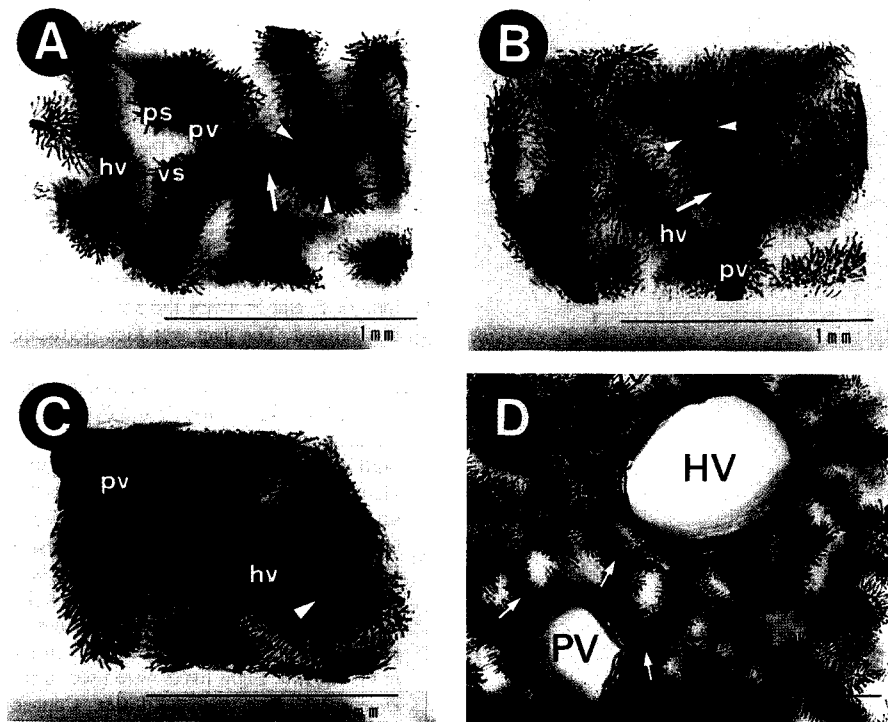
**Fig. 3.** Dual staining of the IPRL with R-6G and acridine orange (AO). The IPRL was infused with 50 nmol/liver of R-6G portally, to increase fluorescence under blue light illumination, and then infused with the same amount of AO in a retrograde fashion. In E and F, twice the amount of AO was infused. The liver was then perfusion-fixed in the antegrade direction and processed as usual. Green excitation revealed only R-6G-stained areas (B and F, red background), while blue excitation visualized both areas stained by AO and R-6G (other pictures, green background). A and B: The same cross section of L-2, 50- $\mu$ m-thick. C: Cross section of the margin of L-2, 50- $\mu$ m. D: Surface microscopy. E and F: The same cross section of a deeper portion of L-1, 200  $\mu$ m. G: Horizontal section of the anterior portion of the left lobe, 200  $\mu$ m. H: Same as in G, 500  $\mu$ m. Eight livers were used in this experiment. Bars indicate 1 mm. Connections between periportal (R-6G-stained) and perivenous (AO-stained) areas can be clearly seen. In the inner portion of the liver, sinusoidal flow directs towards interdigitating terminal hepatic venules as well as larger hepatic vein branches (hv) (A). In the margin of the liver, a fountain-like sinusoidal flow (pv) runs into the terminal hepatic venules both on the top (C and D) and by the side (C). At the hilar portion (E and G), small portal vein branches (pv) that sprouted from large portal veins (PV) interdigitate alternately with small hepatic veins (hv) running into larger veins (HV). The large hepatic vein has three types of branches: large veins (asterisk), small to medium sized vertical veins (arrows), and fine veins that run along the wall (arrowheads) (H). Five livers were used.

ner by 10 and 100 nM prazosin, a selective  $\alpha_1$ -adrenergic blocker, but not by 100 nM atropine (Fig. 5B). In the following experiments, livers were stimulated at frequencies of 10 Hz or less.

On continued stimulation, the elevated PP declined, and this effect was rapid at high stimulation frequencies (Fig. 6A). Infusion of 1.25  $\mu$ M R-6G for 1 min only slightly enhanced the natural decline of the elevated PP at 10 Hz (Fig. 6B). By lowering the frequency to 5 and 2 Hz, the

natural decline became slower and its enhancement by R-6G tended to be greater (data not shown).

Uneven staining became obvious in a frequency-dependent manner (Fig. 7, A–C). At 10 Hz, strong fluorescence was localized around large PVs in the inner portion of the liver, whereas the periphery of the liver showed minimal staining (C–F; compare with Fig. 1, A–D). Horizontal sections revealed extensive staining along large PVs (G and H; compare with Fig. 1, E and F). At higher magnifica-



**Fig. 4.** 3-D flow structures of some portions of the liver reconstructed from dual-stained IPRL. Reconstructed from serial horizontal (A) and cross (B–D)-sections of IPRL dual-stained with R-6G and AO as described in the legend of Fig. 3. Three left lobes were used. The inflow route is designated as dark-painted portal vein branches (pv) with periportal sinusoids (ps, red thin lines) and the draining route as dark-green hepatic vein branches (hv) with perivenous sinusoids (vs, blue thin lines). The space between them should be connected by sinusoids. Intricate interdigitations of portal and hepatic venous branches with their connecting sinusoids can be seen at different portions of the liver. A, Convex surface: view from the top. Arrowheads and arrows indicate final portal vein branches and their pre-branching segments, respectively. B, Convex surface: view from the side. C, Longitudinal edge: view from the side. An arrowhead indicates a final portal vein branch that burrowed to the utmost edge of the liver. D, Hilar portion of the liver: view from the side. Small and short branches (white arrows) sprouted from a portal vein trunk (PV) interdigitate with small hepatic vein branches (black arrows) that are directly connected to the hepatic vein trunk or its large branch (HV).

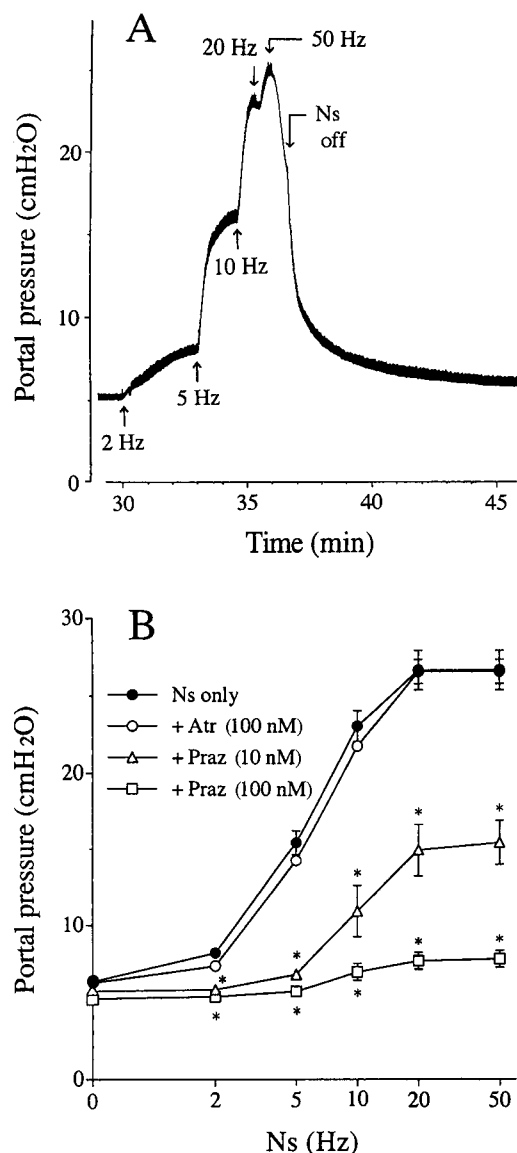
tions (Fig. 8), the staining was widespread from periportal to perivenous areas and occasionally continued to HV branches, as shown in cross sections (A–C) and more clearly in horizontal sections (D–F, arrowheads indicate biased staining toward HVs; compare with Fig. 2). These observations indicated that the sinusoidal flow around the short branches sprouting from large PVs was increased. Slight amounts of R-6G were recovered in the effluent perfusate at 5 Hz ( $0.26 \pm 0.04\%$ ,  $n=5$ ) and at 10 Hz ( $0.84 \pm 0.03\%$ ,  $P<0.05$ ), but none was observed at 2 Hz ( $n=5$ ) or in control IPRLs. Hepatic oxygen consumption was decreased to  $38.2 \pm 1.7\%$  ( $n=10$ ,  $P<0.05$ ) at 10 Hz.

## DISCUSSION

In the present study with control IPRL, portal infusion of R-6G exhibited selective staining of the periportal area, indicating that this lipophilic dye can be efficiently extracted by hepatocytes at proximal portions of sinusoids. This allows indirect detection of perfused sinusoids. R-6G was reported to accumulate in mitochondria and also, at high concentrations, to stain cytoplasmic membranes of cells (16). Recently, it has been widely used to visualize the movement of leukocytes in the microvasculature in vivo

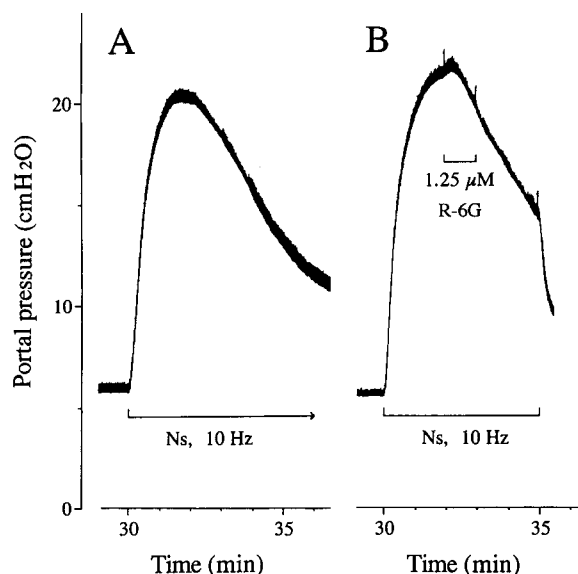
(17–19). Vital staining of the liver with AO has been performed to examine zonal uptake mechanisms in the liver acinus (15) and to observe sinusoidal events on the liver surface under epi-fluorescence microscopy (20, 21). We also used this dye in a previous study (14). However, direct effects of these dyes on liver function are unknown. In the present study, R-6G had no effect on the PP or oxygen consumption in control IPRLs, but in the stimulated livers, it caused a concentration-dependent decrease of the elevated PP, which was greater with AO (preliminary experiments). Such an effect of the fluorescent dye by itself may further alter the flow disturbance by nerve stimulation. This is why we used R-6G, at a low concentration of  $1.25 \mu\text{M}$  for 1 min, in this study. Furthermore, R-6G gave better staining contrast and less decoloration under fluorescence microscopy. It is noteworthy that appropriate dye concentrations should be used to eliminate the effects of the dye itself on experimental systems.

Vital staining of control IPRL with R-6G revealed homogeneous flow throughout the liver in agreement with the intricate branching of PV and, in addition, an acinar type of flow in sinusoids. Analysis of the thick sections of the livers dually stained with R-6G and AO and the reconstructed 3-D flow models indicated that inlet hepatic ven-



**Fig. 5.** Increase of portal pressure by hepatic nerve stimulation and effects of prazosin and atropine in IPRL. A: The hepatic nerve plexus was stimulated on the bifurcation of the extrahepatic portal vein into the median and left lobes by successive increases of the frequency at 20 V/2 ms. B: Infusion of prazosin (Praz) or atropine (Atr) was started 5 min before nerve stimulation (Ns). The stimulation frequency was increased as in A. Values are means  $\pm$  S.E.M. ( $n=5$ ). \*Significantly different from "Ns only" group at  $P<0.05$ .

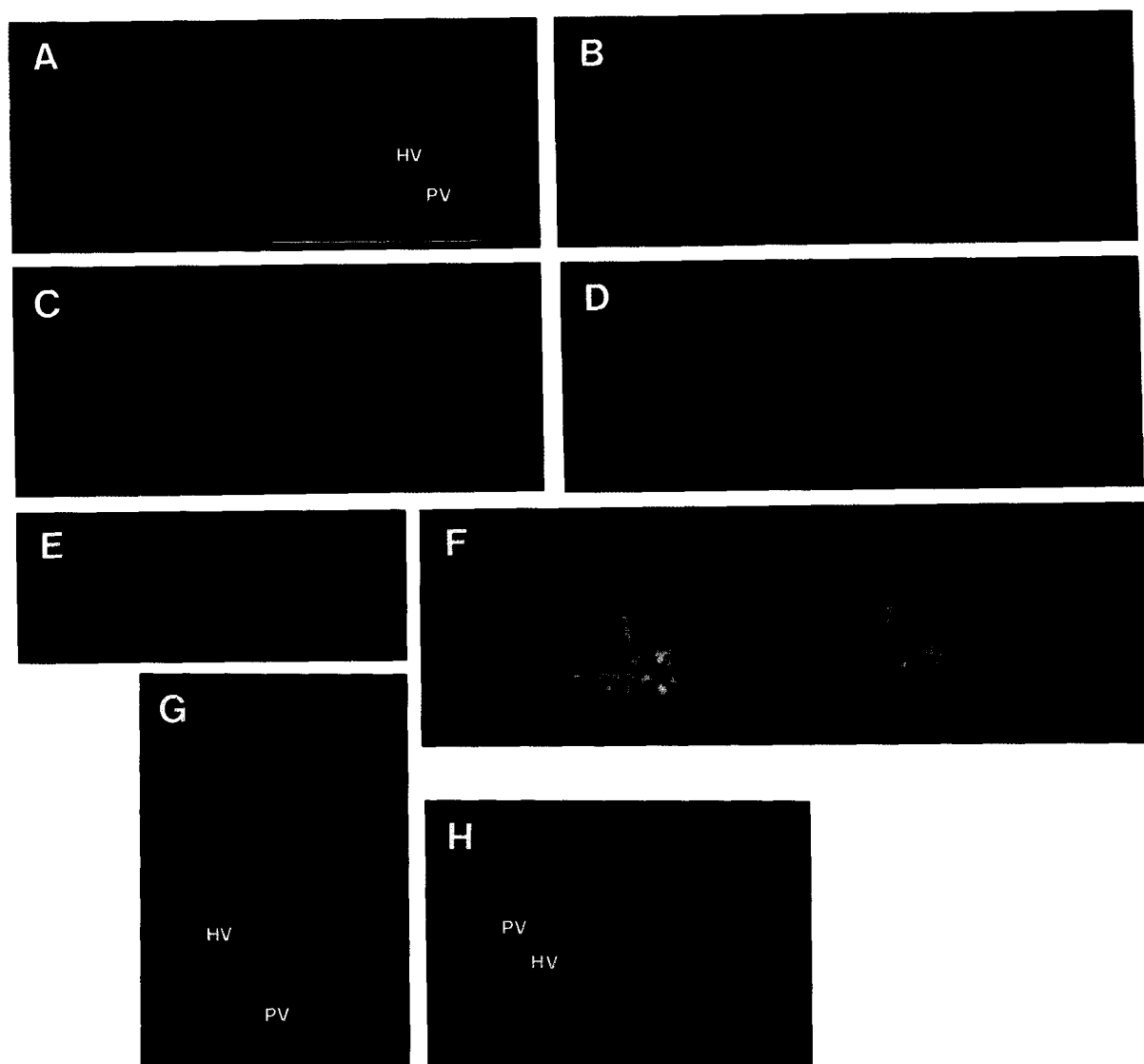
ules to sinusoids have characteristic features for different portions of the liver; fountain-like flow at the surface margin, bending of terminal portal venules into the edge, and the presence of small and short branches that sprouted from large PVs at deeper portions of the liver. The sinusoidal flow appeared to drain rather freely and directly into HVs of various sizes, with an approximate sinusoidal length of 400  $\mu$ m throughout the liver, although the HV trunk received sinusoidal flow through the small branches.



**Fig. 6.** Natural decline of the elevated portal pressure on prolonged nerve stimulation in IPRL (A) and effects of rhodamine 6G (R-6G) infusion (B). A: Perivascular nerve stimulation (Ns) was continued at 10 Hz, 20 V and 2 ms. B: R-6G (1.25  $\mu$ M) was infused for 1 min at the maximum level of portal pressure (stimulated as in A).

There was no staining pattern that suggested a lobular unit. These observations were in accordance with the anatomical angio-structure (22–25).

Hepatic nerve stimulation has long been known to produce metabolic and hemodynamic alterations in the liver accompanied by an increase in PP (26, 27). In the present study, the PP increase was blocked by prazosin but unaffected by atropine. This is consistent with the earlier report (27, 28), confirming that the PP increase is mediated by norepinephrine released at sympathetic nerve terminals without participation of parasympathetic excitation. Since high-frequency stimulation greater than 20 Hz caused a rapid decline of the increased PP, which is probably due to a depletion of releasable norepinephrine, effects on flow distribution were examined at 10 Hz or less, within the physiological stimulation range (26). The idea of flow redistribution during stimulation was originally proposed by Daniel and Prichard (3, 4) by means of radioangiography and later by other investigators using various methods such as those described in the Introduction (5–9). The present study enabled a finer examination of the flow redistribution. Nerve stimulation caused mal-perfusion in the periphery but extensive perfusion in the deeper portion of the liver: the portal flow was short-circuited from the small and short PV branches of large PVs to HV branches, as evidenced by the extensive R-6G staining in this portion. Such a short-circuited flow in the liver was further supported by the leakage of R-6G into the effluent perfusate and



**Fig. 7.** Alteration of the R-6G staining pattern during hepatic nerve stimulation in IPRL (low magnification). Hepatic nerves were stimulated at 2–10 Hz. R-6G (25 nmol/liver) was infused when portal pressure increased to a peak. A–F: Cross sections (50  $\mu$ m) of the liver stimulated at 2 Hz (A, L-1), 5 Hz (B, L-1) and 10 Hz (C, L-1; D, L-2; E, L-3; F, M-2). At each stimulation frequency, 5 livers were used. G and H: Stimulated at 10 Hz, 50- $\mu$ m horizontal sections of the anterior and posterior parts of the left lobe, respectively. Representative results from 3 livers are shown. The bar indicates 1 cm. Heterogeneous staining became demarcated as the stimulation frequency increased (A–C). The staining was concentrated in the inner part of the liver mass, particularly around the large portal veins (PV), while the periphery or edge of the liver was poorly stained (C–H). Compare with Fig. 1.

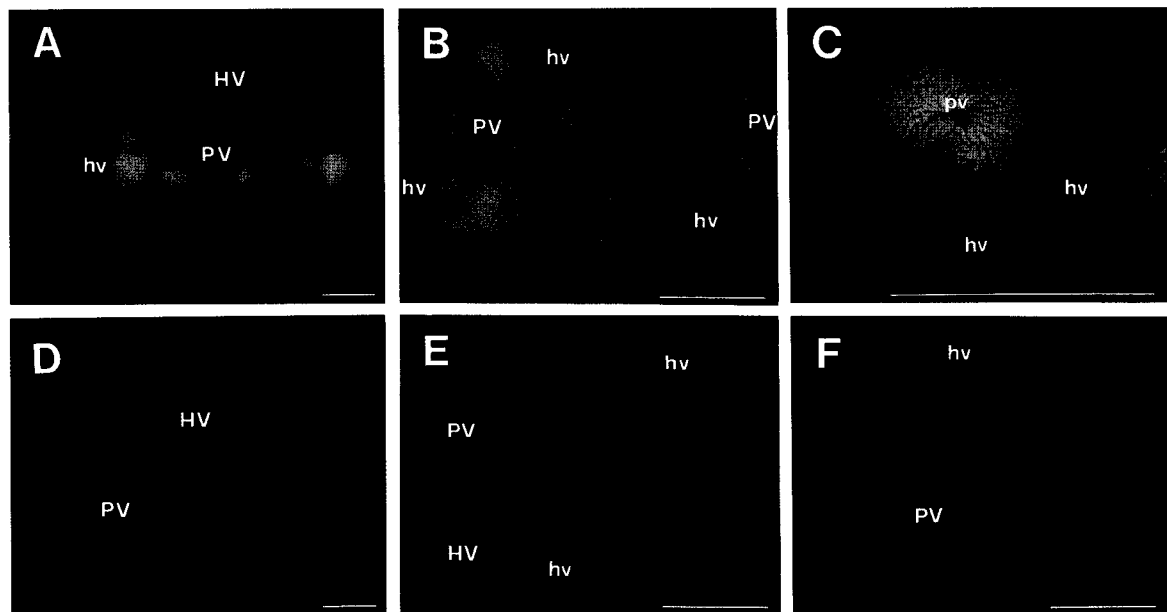
also by a decrease in hepatic oxygen uptake. The flow redistribution may be due to effective contraction of peripheral portal branches and subsequent increase of pressure in large PVs. However, the precise sites of contraction remain to be elucidated. We speculate that the short-circuited pathway could serve as an anatomical device to control unusually high PP.

Thus, the method described here can finely visualize not only sinusoidal perfusion but also vascular networks that give flow to sinusoids, from surface to the deeper portion

of the liver. For this, in addition to observations on thin cross sections which were examined in our previous study (14), thick sections cut in different directions or surface microscopy are useful.

The method may be applied to examine the effects of vasoactive substances on intrahepatic flow distribution, as reported with endothelin-1 (14, 29) and U-46619 (thromboxane A<sub>2</sub> analog) (14). In contrast, when considering metabolism in the liver, it is essential to eliminate the possibility of flow redistribution. For example, various meta-





**Fig. 8.** Alteration of the R-6G staining pattern during hepatic nerve stimulation in IPR (high magnification). The hepatic nerves were stimulated at 10 Hz. A – D: 50- $\mu$ m liver sections obtained from the experiments shown in Fig. 7. A and C: Cross section (L-1). B: Cross section (M-2). D: Horizontal section of the anterior part of the left lobe. E and F: Representative sections (500- $\mu$ m) obtained from 5 additive experiments are shown (the same portion as in D). Bars indicate 1 mm. In both cross and horizontal sections, extensive staining spread from the short branches of large portal veins (PV) toward the perivenous side and occasionally continued to hepatic vein branches (hv) of various sizes (arrow heads), indicating increased flow in this area (compare with Fig. 2, D – F).

bolic effects of oxethazaine (topical anesthetic) (30) were found to be due to flow redistribution (14). Flow disturbance under pathological conditions *in vivo* may also be studied using this method; e.g., cirrhotic liver (29).

In conclusion, the intrahepatic flow distribution was examined in detail by vital staining of the liver with R-6G, perfusion-fixation and subsequent fluorescence microscopy of the sections. The method enables demonstration of perfused sinusoids as well as the vascular networks that give flow to sinusoids. Comparing with the other methods, the present method can visualize real intrahepatic flow at any portion of the liver and is relatively simple, and thus, it may be useful for examining intrahepatic flow disturbance under various conditions or to test the effects of various factors on intrahepatic flow.

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