THE ESTIMATION OF HEPATIC BLOOD FLOW USING INDOCYANINE GREEN AND GALACTOSE

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The University of Manitoba

In Partial Fulfillment of the Requirements for
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by

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ABSTRACT

Experiments were performed to determine the validity of the indocyanine green (ICG) and galactose clearance techniques, with and without allowances for incomplete hepatic extraction, as an estimate of hepatic blood flow. These techniques were compared to that of directly measured hepatic blood flow using a hepatic venous long-circuit preparation in the anesthetized cat. This preparation allowed direct measurement and alteration of hepatic blood flow, and collection of arterial, portal and hepatic venous blood samples without depletion of the animal's blood volume. Measurements of ICG by the spectrophotometric and high pressure liquid chromatographic (HPLC) methods were equally accurate but the HPLC method was 40 times more sensitive and allowed smaller sample volumes. It was determined that systemic clearance of ICG after a bolus dose (1.3 μmol/kg) markedly underestimated true hepatic blood flow. Correction must be made for the incomplete extraction. When the clearance was corrected for extraction, mean estimated hepatic blood flow exceeded the measured flow values by some 20%. In all experiments estimated hepatic blood flows were highly variable. ICG was found to be distributed into extrahepatic tissues in hepatectomized cats. This may explain part of the overestimation of hepatic blood flow.

Systemic clearance of ICG during a 3.22 nmol/kg/min infusion was unreliable in estimating hepatic flow. When

systemic clearance was corrected for the incomplete extraction, mean estimated hepatic flow initially overestimated the true flow by 53%. Reasonable mean estimates of flow were obtained only after 50 min of infusion, but data obtained from any one cat were highly variable. When hepatic flow was altered in either direction, the mean estimated flow underestimated the change in measured flow by 12%-15%.

Reliable estimates of hepatic blood flow using infusions of galactose were not obtained. Infusion rate could not be used as an estimate of hepatic or splanchnic uptake due to substantial and variable extra-splanchnic uptake. As a result, estimated hepatic flows allowing for incomplete extraction over-estimated the true flow. On the other hand, extraction was less than 100%. This caused systemic galactose clearance to underestimate hepatic blood flow. These errors could cancel each other giving an apparently good estimate of hepatic flow from systemic galactose clearance. This agreement was fortuitous and occurred only at a specific dose and blood flow. We conclude that in the absence of independent measurements of both extra-splanchnic uptake and splanchnic extraction of galactose, systemic galactose clearance is not a reliable measure of hepatic blood flow in anesthetized cats. Until proven otherwise, it seems likely that this is also true in humans.

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INTRODUCTION

A. Hepatic Circulation

1. General

The splanchnic vascular bed is a large blood reservoir which serves important functions in the overall homeostatic adjustments of the circulatory system. Within this bed lies the largest gland in the body, the liver. The liver represents only 2-3% of total body weight, yet it is perfused with approximately 25% of the cardiac output. The liver receives a total of 100-130 ml blood/min/100g liver (30 ml/kg body weight) in the dog, cat and human. Oxygen rich blood is supplied by the hepatic artery and represents 20-33% of the total blood supply to the liver. The remainder of hepatic blood flow (67-80%) is supplied by the portal vein. The portal vein in turn receives its blood supply from the spleen (10%), pancreas (10%), stomach (20%) and intestine (60%). The relative contributions of the blood supply vary depending on the physiological state. Vascular pressures for the hepatic artery are about 100 mm Hg and 7-10 mm Hg for the portal vein, while sinusoidal pressure is similar to that of the portal vein (63, 66).

2. Microcirculation

The acinus is the microvascular unit of the liver. It consists of a cluster of parenchymal cells grouped about a

terminal hepatic arteriole, portal venule and bile duct. Blood enters the acinus at the center (zone 1) and exits via the hepatic venules of zone 3. This arrrangement does not allow substances to diffuse from zones 3 to 1 (122, 155). However, it is possible that substances which are secreted by the cells of zone 1 may be taken up by the cells of zones 2 and 3. Direct experimental evidence demonstrating uptake of substances by the peripheral cells is lacking.

The hepatic microcirculation originating from both the portal vein and hepatic artery has been described through morphological (39, 73, 134, 138, 142) and transillumination studies (131, 154). The portal vein branches 6-10 times before the venules conduct blood into the sinusoids of the periportal or zone 1 region of the liver lobule. The hepatic artery closely accompanies the portal vein as it branches and penetrates the parenchyma (32, 70). Scanning electron microscopy of rat liver vascular casts illustrates that when the liver is perfused via the hepatic artery, the portal vein, peribiliary plexus and sinusoids are also perfused. The hepatic artery was found to have its most numerous connections with the peribiliary plexus (70). The majority of the hepatic arterial connections with the portal vein occur at the inlet portal venules. Connections with the larger portal branches also exist in the rat

(138).

Vascular casts of rat liver, prepared by perfusing the portal vein, show that the portal vein does not perfuse the hepatic artery and only poorly perfuses the peribiliary plexus (138). The portal vein is, however, connected to the hepatic artery and peribiliary plexus by small vessels. Grisham and Nopanitaya (70) suggested that the hepatic artery may perfuse the portal vein but the portal vein does not perfuse the hepatic artery, since the artery is not appreciably filled when the portal vein is perfused (70).

Recent observations have been made concerning the hepatic arterial terminations in the rat, hamster and human livers by scanning electron microscopy of microvascular casts (89, 204). The hepatic arterioles were found to anastomose with the terminal portal venules while others anastomosed with the inlet venules. A few hepatic arterioles terminated in sinusoids adjacent to the portal tracts. These vessels shared a common entrance into the sinusoids with the inlet venules. Arterioportal anastomoses were found to be present in the mouse (70) and rat (142) livers. However, arterioportal anastomoses were not found in either hamster or human livers. The terminal hepatic arterioles, in these species, are connected directly to the sinusoids (89, 204). Arterioportal

anastomoses were also not found in either monkey (136) or rabbit (70) livers. The unique architecture of the rat liver leads to some concern about the use of this species in studying the hepatic circulation since the anatomy is unusual if not unique (204).

The peribiliary plexus consists of a network of capillary vessels that surround the bile ducts. Results from studies with injected dyes (41, 73, 134) and vascular casts (70, 138, 142) in rat liver suggest that the major afferent vessels which coil around the bile ducts originate from the hepatic artery. In the periphery of the liver lobe, the biliary plexus consists of a sparse capillary network around the small bile ductules. The efferent vessels of the peribiliary plexus are a source of blood to the sinusoids, especially in the perihilar regions of the liver. Thus, all the blood flowing to the liver passes through the sinusoids. Some connections of the peribiliary plexus were also found with the small portal veins (70, 138, 142).

Information obtained through the use of vascular casts, with respect to the arterial and portal microvessels, can only be assessed qualitatively. The methodology does not allow quantitative assessment regarding the fluid fraction derived from the hepatic

artery or portal vein. To determine the distribution of hepatic arterial and portal flow within the liver, Greenway and Oshiro used radioactive microspheres (64). Their study demonstrated that mean flow/g to any lobe or segment of a lobe was similar to the mean flow/g of the whole liver. Generally, the liver was found to be homogeneously perfused by both portal and arterial blood in anesthetized cats and dogs. These findings, obtained with microspheres, were similar to those obtained using labelled water (68, 129).

Other studies have similarly found that the sinusoids are perfused uniformly by arterial and portal blood. Administration of taurocholate into either the hepatic artery or portal vein in anesthetized cats increased bile flow to the same extent (119). The elimination of various other substances administered by either route was also found to be similar (11, 16, 29, 121). Furthermore, hepatic nerve stimulation has been shown not to alter flow distribution as assessed by microspheres (65), lidocaine (108) and oxygen uptake (111). Similar results were obtained when hepatic venous pressure was raised (64, 112). However, histamine, in the dog, produced some redistribution of portal blood flow towards the hilar regions (64).

B. REGULATION OF HEPATIC BLOOD FLOW

1. The Hepatic Arterial Buffer Response

One of the first reports suggesting that total hepatic blood flow remains constant was by Burton-Opitz (18). He reported an increase in hepatic arterial flow at a time when the portal venous flow was obstructed. Much later, Greenway and Stark (63) reported that a decrease in portal flow may be accompanied by an increase in hepatic arterial flow. As portal flow to an area of the liver was reduced, e.g. by occlusion of a branch of the portal vein, that area received a greater supply of arterial flow (64). Total hepatic blood flow, therefore, remained within a constant range.

The function of the hepatic artery was proposed to prevent large irregular changes in hepatic blood flow. The significance of this control was thought to maintain the clearance rates of endogenous substances constant (e.g. hormones). Lautt regarded the hepatic artery as the guardian of normal hepatic clearance rates of humoral substances (109).

Various hypotheses have been proposed to account for the hepatic arterial and portal venous blood flow interaction. The metabolic hypothesis states that if

tissue oxygen supply does not meet the metabolic demands of the hepatocytes, vasodilator metabolites are released and diffuse to the resistance sites causing vasodilation. This hypothesis suggests that the metabolites diffuse from the peripheral cells of the sinusoid against the flow of blood to the vascular resistance sites. The proposed myogenic hypothesis states that an increased transmural pressure gradient across the arterioles causes increased stretch of the vascular smooth muscle cells. These cells respond by contracting. The contraction causes an increase in vascular resistance, thus maintaining a constant hepatic blood flow (117, 122, 168).

The metabolic mechanism for control of total hepatic blood flow may be discarded based on hemodilution studies performed in anesthetized cats (110). Hemodilution resulted in decreased oxygen delivery to the liver. The gut responded by vasodilating which caused portal flow to increase. During this time the hepatic arterial flow decreased thus, maintaining total hepatic blood flow constant. The degree of hepatic arterial flow reduction correlated with the increase in portal flow. Studies were also performed in order to change oxygen demand by the administration of dinitrophenol (DNP) to stimulate liver metabolism and SKF-525A to inhibit the hepatic metabolism of other endogenous substrates. The gut responded to DNP

by vasodilation causing the hepatic artery to constrict. Similar results were obtained for SKF-525A (116). These studies suggested that the hepatic artery is not controlled by the metabolic demands of the liver.

There is some evidence to support the lack of a myogenic mechanism. The hepatic arterial blood flow has been shown to be inversely related to portal venous blood flow; i.e. as portal flow decreases, hepatic arterial flow increases. No correlation was found between hepatic arterial blood flow and either portal or arterial pressures. This suggests that the hepatic arterial response to the change in portal venous flow does not involve a myogenic mechanism. However, a myogenic mechanism may still exist. As hepatic venous pressure increases, hepatic arterial blood flow remains constant despite a decrease in portal venous blood flow. The myogenic response of the hepatic artery to the raised hepatic venous pressure may have countered the expected arterial response in this case. Overall, the intrinsic control of hepatic arterial blood flow would seem to be designed to keep total hepatic blood flow constant and independent of either metabolic or myogenic control (117).

The relationship between the hepatic arterial and portal venous blood flow has been termed the hepatic arterial

buffer response. This mechanism is currently generating much discussion. It was proposed that the hepatic arterial smooth muscle produced a dilator substance (117). This substance can either increase arterial blood flow through vasodilation of the artery directly or it can be washed away by the flowing blood supply thus exerting no effect. By this mechanism, portal blood must have access to the vasodilator release site(s). During periods of low portal flow the hepatic artery dilates. Conversely, during periods of high portal flow the hepatic artery constricts. The vasodilatory substance was recently proposed to be adenosine (123). The eligibility of adenosine to be the dilator substance controlling the buffer response included studies showing that; 1) adenosine dilates the hepatic artery; 2) portal blood has access to the arterial resistance vessels such that the metabolite can be washed away during periods of high portal blood flow; 3) exogenous adenosine agonists potentiate the buffer response; and 4) exogenous adenosine antagonists inhibit the buffer response (118, 123). Data supporting the contention that adenosine is in fact responsible for the buffer response have been reviewed (66, 122). However, definitive experiments demonstrating that the local concentration of adenosine is actually regulated by washout in the face of a constant production are lacking.

2. Neural Control of Hepatic Blood Flow

The hepatic blood vessels are innervated by the anterior and posterior hepatic plexuses which contain both sympathetic and parasympathetic fibers. The anterior plexus forms a sheath along the common hepatic artery while the posterior plexus branches to the portal vein and bile duct. These nerve bundles meet at the junction of the hepatic and gastroduodenal arteries and pass to the liver. Stimulation of the nerves causes an increase in inflow resistance of both the hepatic artery and portal vein. The increased resistance of the hepatic artery results in a lower total hepatic blood flow (57, 69, 115, 120) while the increased resistance of the portal vein serves to elevate portal pressure. It in itself does not decrease portal blood flow. The reduction in total hepatic blood flow is also not associated with an intrahepatic redistribution of arterial blood (65, 166). In the cat, continued stimulation of the hepatic nerves produces a phenomenon known as autoregulatory escape. In this state the hepatic arterial flow returns toward the initial flow rate despite the continued nerve stimulation. During nerve stimulation there is a concomitant reduction in hepatic blood volume. The reduction in blood volume is maintained throughout the nerve stimulation period in both cats (60) and dogs (20). The liver is, therefore, acting as a blood reservoir which can be mobilized through the sympathetic innervation. The

hepatic arterial constriction to nerve stimulation can be reversed by the administration of alpha-adrenoceptor blockers (57). The effect of cholinergic vagal stimulation appears to be minimal. Although in one study using a transilluminated rat liver, vagal stimulation dilated the calibre of liver sinusoids. The dilation opened previously closed sinusoids such that the total number of perfused sinusoids increased (98, 99, 100). Conversely, other investigators have found no change by such stimulation (for review see refs 63, 120).

3. Effect of Drugs and Hormones on Hepatic Blood Flow

Many studies and reviews address the effect of drugs and hormones on hepatic blood flow (63, 162, 163, 169) and will be discussed only briefly. It should be noted that studies which show a direct comparison between the plasma or blood concentrations of the various substances and their effect on liver blood flow are lacking. Most studies give data in terms of the amount administered. There is very limited knowledge concerning effective blood concentrations and the proposed pharmacological response. Since hepatic blood is supplied from two sources, viz. hepatic artery and portal vein, the effects of substances derived from gastrointestinal origin will be discussed first.

a. Effect of Meals on Hepatic Blood Flow

Portal and systemic osmolarity is known to increase as a result of absorption of a meal from the gastrointestinal tract (21). The increase in osmolarity increases intestinal blood flow due to a reduced vascular resistance (128). It follows that the postprandial increase in osmolarity would be expected to give rise to an increase in portal venous blood flow. Conscious dogs fed a meat meal have been shown to have a 32% increase in total hepatic blood flow. The increased flow, which remained elevated for 3-4 hr, was attributed to intestinal vasodilation causing an increased portal blood flow. Hepatic arterial flow remained unchanged (78). A high fat meal also produces circulatory changes in animals. A butter-fat meal is associated with increased adhesiveness and aggregation of the red blood cells. The alteration in the rheological properties of the red blood cells were accompanied by a slowing of the circulation in rabbits and dogs (192). In humans, splanchnic blood flow is not affected after a high glucose meal (15, 26). The extent (if any) to which hepatic blood flow is changed after a high fat or high glucose meal is not known.

b. Effect of Bile Acids on Hepatic Blood Flow

The effects of exogenous bile salts and bile acids on

hepatic blood flow differ widely with the substances and doses being assesed. Sodium dehydrocholate, for example, increases hepatic arterial blood flow unless the systemic arterial pressure falls. The decreased arterial pressure in this case counteracts the increase in blood flow. The net result is a relatively constant hepatic blood flow. Portal blood flow usually decreases but, again, it is highly variable (52, 151). In man, hepatic blood flow increases as estimated by the clearance method (133). Hydrocholeretics tend to increase hepatic arterial blood flow but substances which increase the secretion of bile solids (conjugated cholates) do not have any marked effect on total liver blood flow (52).

c. Effect of Gastrointestinal Hormones on Hepatic Blood Flow

Gastrin and pentagastrin, vasoactive intestinal polypeptide, secretin, cholecystokinin-pancreozymin, insulin and glucagon are among some of the expanding list of substances found to be released by the splanchnic organs into the portal venous blood. Generally, these substances tend to increase hepatic blood flow. The doses used to induce this increase tend to be much greater than that encountered physiologically (54, 66, 151). Furthermore, it is not known at which blood concentration the resulting

physiological response is elicited. Of the various gut hormones studied, cholecystokinin-pancreozymin increased hepatic blood flow at doses that may be encountered in the postprandial state (54). Glucagon infused intraportally increased superior mesenteric blood flow in a dose-dependent manner (167). Hepatic arerial blood flow, at this time, decreased slightly. The increased portal flow, together with the decreased arterial flow, was thought to be the result of the buffer response (66). Insulin, administered intravenously or intra-arterially at a dose of 1.0 U/kg, increased total hepatic blood flow in dogs. The increased flow was suggested to be due to the adrenaline release induced by hypoglycemia. At physiological concentrations, however, insulin is without effect on hepatic blood flow (184).

d. Effect of Adrenergic Drugs on Hepatic Blood Flow

Numerous studies have led to the understanding that both the hepatic artery and portal vein possess a substantial alpha-adrenoceptor population mediating vasoconstriction. Intra-arterial administration of noradrenaline decreases hepatic arterial flow in cats (57, 173), dogs (77, 161, 165) and monkeys (191). The vasoconstrictor action of noradrenaline on the hepatic artery is blocked by the alpha-adrenoceptor antagonists

phenoxybenzamine (77), dibenamine (71, 77) and phentolamine (164). Blockade of the beta-adrenoceptors with propranolol increases the vasoconstrictor potency of noradrenaline (72, 173). Autoregulatory escape also occurs from the portal vasoconstrictor action of noradrenaline similar to that produced through hepatic nerve stimulation (71). Intraportal administration of noradrenaline produces similar effects as those administered intra-arterially. The portal responses are not affected by beta-adrenoceptor blockade but are antagonized by the alpha-adrenoceptors (169). Noradrenaline administered intravenously also reduces total hepatic blood flow in the dog (35, 84, 194), rabbit (55) and cat (7, 42, 56). Thus, the vascular effects of noradrenaline are the result of an interaction largely with the alpha-adrenoceptors and to a lesser degree with the beta-adrenoceptors.

Experimental evidence suggests that the hepatic arterial resistance site possess a substantial beta-adrenoceptor population mediating vasodilation. These receptors are not present to any significant degree in the portal vascular resistance sites. There is no direct response to isoproterenol administered intraportally nor does the systemic administration of propranolol modify the adrenaline or noradrenaline induced vasoconstriction. The beta-adrenoceptor population was found to be predominantly

of the $beta_2$-subtype, since they are stimulated by salbutamol and not by dobutamine, and are blocked by propranolol but not by atenolol (164, 169). The hepatic vasculature is also populated by dopamine receptors. When stimulated these receptors cause vasodilation which can be blocked by haloperidol. The portal vessel, however, does not possess a significant dopamine receptor population (169).

Adrenaline produces similar effects to those of noradrenaline when infused into the hepatic artery or portal vein (2, 63). Adrenaline administered intravenously increases total hepatic blood flow with little change in mean arterial pressure in the cat (56), dog (8, 42, 187) and man (7). The increase in flow was suggested to be due to intestinal vasodilation mediated by the beta-adrenoceptors (63). The vascular effects of adrenaline are modified by propranolol and phenoxybenzamine resulting in vasoconstriction and vasodilation, respectively (57, 173).

e. Effect of Cholinergic Drugs on Hepatic Blood Flow

Intra-arterial administration of acetylcholine causes the hepatic artery to dilate provided that it is not maximally dilated before drug administration (2, 22, 179).

The vasodilator response is blocked by atropine (2, 169). Acetycholine administered intraportally can either increase (2) or have no effect (179) on portal vascular resistance (169). If portal vascular resistance increases the hepatic artery dilates (2, 179). The dilation may be due to either the buffer response or a direct effect of acetylcholine on the hepatic artery.

f. Effect of Autacoids on Hepatic Blood Flow

In man, intravenous infusions of bradykinin produced a 10% increase in total hepatic blood flow as estimated by indocyanine green clearance data (44). The majority of the increased flow may be due to an increase in portal venous flow via intestinal vasodilation (54). In the dog, bradykinin is the most potent hepatic arterial vasodilator (163, 179). Serotonin causes a weak and variable response in the dog liver vasculature (169). Angiotensin decreases total hepatic blood flow through a decrease in both arterial flow (91, 179) and portal flow (6, 27, 59, 62, 132) through intestinal and splenic vasoconstriction (36). Similarly, vasopressin decreases total hepatic blood flow through intestinal and splenic vasoconstriction (27, 54, 62, 148, 157). Various hormones and autacoids have been demonstrated to dilate the hepatic artery. Some of these include; parathyroid hormone (23), prostaglandin E_2 and

histamine (162, 163). In the cat, histamine administered intravenously reduces hepatic arterial blood flow but increases portal venous flow (101). This was suggested to be the result of the hepatic arterial buffer response over-riding the direct effect (66). For further reviews on this topic see Greenway and Stark (63), Richardson and Withrington (168, 169) and Greenway and Lautt (66).

g. Effect of Aging on Hepatic Blood Flow

Basic animal experiments elucidating the effect of age on hepatic blood flow and hepatic function are lacking. Aging is known to be accompanied by a number of hemodynamic changes (9). The vascular components of the peripheral circulation are affected asymmetrically. The cerebral, coronary and skeletal muscle circulations are minimally affected since they receive a greater proportion of the decompensated cardiac output. The splanchnic and renal circulations show a decreased flow which surpasses the decreased cardiac output (9). Hepatic blood flow has been estimated to decrease at 0.3% per year based on normal flow values and average liver weight change with age (104). A reduction of 1.5% per year in total hepatic blood flow was suggested from a study by Sherlock, et al. (183), using the clearance technique in male subjects. The data suggest, that at age 65, hepatic blood flow might be expected to be

reduced to 40-45% compared to that at age 25 (9). The effect of diseases or drugs on a decompensated vascular bed is not known.

Studies which examine the effect of aging on hepatic blood flow use the clearance technique for estimating total hepatic blood flow. The clearance of indocyanine green (203), lidocaine and propranolol (182) have been shown to decrease with age. The decreased clearance of these substances is generally accepted as being indicative of a decrease in hepatic blood flow since they are highly extracted (201). However, liver size has been documented to decrease with age in humans (94). Thus, the decreased clearance of these substances may be due to a decrease in liver size. Total hepatic blood flow per 100g liver in the elderly may, in fact, be similar to that of the young. Furthermore, it is not known whether the clearance technique yields reliable data (see later).

C. ESTIMATING TOTAL HEPATIC BLOOD FLOW

Measuring blood flow is of fundamental importance to the understanding of the physiology of an organ or vascular bed. Such knowledge is of benefit in furthering our

understanding not only of the influence that the various disease states may have, but also the effect that drugs may have upon the vascular bed. Knowledge of hepatic blood flow is also of importance in the clinical situation, e.g. the management of portal hypertension. Hepatic blood flow was found to be a useful guide in the selection of patients for portacaval shunting procedures (198). In attempting to quantitate total hepatic blood flow the hepatic vascular bed possess a unique problem to investigators. The liver, with its dual blood supply, requires two flow measurements. Quantitating hepatic venous outflow would circumvent the need for two measurements but the short length of the hepatic vein and its anatomical position relative to the inferior vena cava, diaphragm and liver make this measurement impractical.

Methods for measuring total hepatic blood flow can be classified as either direct or indirect. Direct techniques are invasive, while the indirect techniques may be either invasive or noninvasive.

1. Direct Methods

a. Timed Collection

The most reliable method available to directly measure total hepatic blood flow is a timed collection of mixed

hepatic venous outflow (61). The preparation using this method, called the hepatic venous long-circuit, involves extensive surgery (see Methods). The advantage is that there is no interruption of blood flow and no congestion. Balloon catheters introduced through the jugular (10) or femoral (153) veins have also been used to divert nonhepatic blood. Although these methods are highly accurate their use is very limited. Nevertheless, these techniques are excellent for calibrating other currently available methods.

b. Flowmeters

Another reliable and widely used method for direct measurement of hepatic blood flow in both the clinical and experimental situations uses the square wave electromagnetic flowmeter. The flowmeters work on the principle that the velocity of blood, passing through a magnetic field, generates an induction voltage which is measured between two electrodes, in this case the flowprobe. The induced voltage is directly proportional to the blood flow (96, 97). The advantage of this technique is that it can be used on large or small, cannulated or uncannulated, vessels (141). A disadvantage is that the flowmeters require extensive calibration and repeated zeroing because of baseline drift. The probe should be calibrated using blood during the experimental condition,

since changes in viscosity and geometry alter blood conductivity (130, 170). With proper calibration and repeated zeroing, the electromagnetic flowmeter gives reliable results.

The ultrasonic flowmeter may be considered as the state of the art technique. There are three basic flow probe devices. The pulsed ultrasonic probe uses two piezoelectric transducers attached at 45^{o} angles to opposite ends of the vessel. Each probe alternately transmits and receives signals 800 times/second (87). The Doppler frequency shift flowmeter is similar to the pulsed probe, the difference being that one transducer acts as the transmitter while the other acts as the receiver, thus giving a unidirectional signal (48). The final ultrasonic flowprobe is an implantable and telemetered probe. A single probe is used to transmit and receive signals (1). The ultrasonic flowprobes do not exhibit zero drift which is a major disadvantage of the electromagnetic flowprobes. A disadvantage of the ultrasonic flowprobes is that probe function is assumed to be held constant during the course of an experiment. In situ calibration is also required (1, 87).

2. Indirect Methods

a. Indicator Dilution Technique

The indirect methods for estimating hepatic blood flow are based on the Fick principle introduced by Adolf Fick in 1870 (147). The first method uses indicators which are diluted by the circulating blood supply. The technique, termed the indicator dilution, or dye-dilution technique, is an extension of the Fick principle. It is not dependent on hepatic function and may be more accurate than the clearance method in estimating flow during liver disease. The method measures total hepatic blood flow. Nothing can be stated regarding functional blood flow, i.e. blood perfusing the hepatocytes (79). Substances used for this method include ^{51}Cr - red cells (53, 186) and ^{131}I - albumin (53, 82).

The expression relating blood flow (in this case total hepatic blood flow) to the concentrations of the indicator immediately after it has passed through an organ (liver) is:

$$EHBF = X_o / \int_o^\infty [C(t) \cdot d(t)]$$

where:

EHBF = estimated hepatic blood flow;

X_o = total amount of indicator administered and;

C(t) = concentration of indicator at any time (t).

To use this method properly certain conditions must be met. First, the Fick principle presupposes that blood flow is constant and not pulsatile. Using this principle, it is necessary to postulate that blood flow during sampling remains constant (147, 197). Second, the indicator must remain in the vascular space and must not be metabolized prior to the sampling site. If some of the indicator bypasses the liver through extrahepatic portasystemic shunts, then the concentration lost must be similar to that sampled at the hepatic vein in order to obtain reliable estimates of blood flow (14). Finally, sampling of hepatic venous blood through a hepatic vein catheter must be representative of total hepatic venous outflow.

b. Clearance Technique

A second widely used technique for estimating total hepatic blood flow is based on measuring the clearance of a substance from blood. This method is useful only if the extraction of the test substance by the liver can be quantitated. The technique, termed hepatic clearance, utilizes a variation of the Fick principle. It relates the selective removal of the test substance by hepatocytes to the hepatic blood flow.

Two models have been proposed which relate clearance to blood flow; the venous equilibration model and the parallel tube model. Recently, both models have been reviewed (66) and both are based on several important assumptions. The parallel tube, or sinusoidal perfusion model, regards the liver as composed of a large number of identical 'cylindrical tubes' arranged in parallel with the enzymes uniformly distributed in the parenchymal cells. As blood flows unidirectionally along the length of the sinusoids the concentration of substrate decreases. Elimination, in this model, depends on the Michaelis-Menton relationship. The substrate concentration in the sinusoids is regarded as the logarithmic average concentration of substrate entering and leaving the liver (143).

The venous equilibration model (also known as the well-stirred or equilibrium model) assumes that the distribution of substrate into the liver is perfusion rate limited and that elimination is a first order process (201). Unlike the parallel tube model, the venous equilibration model considers the liver as a single well-stirred compartment. Distribution equilibrium is achieved rapidly such that the drug concentration in the emerging venous blood is in equilibrium with that in the liver. There is no substrate concentration gradient along the sinusoid in this model. This assumption is both

pharmacologically and physiologically untenable and leads to the rejection of this model. However, qualitatively both models predict similar effects of hepatic blood flow on drug clearance.

Hepatic clearance, defined as the apparent volume of blood (or plasma) which is completely cleared of substrate by the liver per unit time, is related to hepatic blood flow and extraction (201).

$$Cl_H = F_H \cdot E$$

where:

Cl_H = hepatic clearance;

F_H = hepatic blood (or plasma) flow;

E = extraction calculated as (Ca-Cv)/Ca;

where:

Ca = arterial substrate concentration and;

Cv = hepatic venous substrate concentration.

Hepatic clearance is, therefore, a function of liver blood flow and the ability of the liver to extract the substrate. A family of curves was described which related hepatic clearance to hepatic blood flow for drugs with various extractions (extractions of 0.1 to 0.9 at normal hepatic blood flow). The lower the extraction the less dependent hepatic clearance is on blood flow and extraction

will be sensitive to changes in flow. When flow changes, extraction changes in the opposite direction. Thus, hepatic clearance should be compensated by an opposing action of extraction and the product of hepatic blood flow and extraction will be relatively constant. Conversely, for substances which are highly extracted (extraction approaching unity), hepatic clearance will be highly dependent on blood flow. The change in extraction does not fully compensate for the change in flow (201).

The clearance method, first described by Bradley, et al. (12), is used most frequently. Dyes or radioactive labeled substances are administered either as a bolus dose or an intravenous infusion. Some of the test substances employed include: bromsulphthalein (12), indocyanine green (19), galactose (76), rose bengal (31) and ethanol (202). These substances are thought to be highly and solely extracted by the liver. Their clearance rates should, theoretically, reflect hepatic blood flow. The clearance technique measures the efficiency of hepatic elimination and is dependent on both the hepatic cell function and the quality of hepatic blood flow (79). In cirrhosis, however, there are anatomical changes in the liver microcirculation. Porto- and/or arterio-hepatic venous shunts and capillarization of the sinusoids occur (14, 150, 176). It is, therefore, uncertain whether this technique is of value

in the clinical or prognostic situation in patients where liver disease is present (160).

Using the clearance method during a constant infusion, after attaining a stable blood concentration, the amount extracted by the liver is equal to the amount infused (assuming no extrahepatic uptake). Estimated hepatic blood flow during steady-state may then be calculated as:

$$EHBF = K_o/(Ca-Cv)$$

where:

K_o = infusion rate.

Note that the denominator in this expression is an indication of extraction efficiency and requires the sampling of hepatic venous blood which, in turn, requires hepatic vein catheterization. Ethically this is more difficult to do. Thus, to overcome this problem extraction of the test substance by the liver is assumed to be 100% during steady-state (assuming no extrahepatic uptake). Thus, the above equation reduces to:

$$EHBF = K_o/Ca$$

where:

Ca = steady-state arterial blood concentration.

Following an intravenous bolus injection the dye is mixed with time by the circulating blood. Together with extraction by the liver, the arterial concentration is progressively reduced. If the dye is confined to the circulating blood volume and is completely removed by the liver (extraction equals 100% during a single pass through the liver), the disappearance rate from plasma is related to the hepatic blood flow. Thus, the equation for estimating hepatic blood flow may be modified for bolus doses.

$$EHBF = CL_S = Cl_H = (Vd \cdot K)$$

where:

Vd = the circulating volume of distribution of the test substance;

K = the apparent first order elimination rate constant and;

Cl_S = systemic clearance.

If the test substance is not completely removed by the liver during a single pass (extraction less than 100% and assuming minimal extrahepatic uptake) then the above equation must account for the incomplete extraction. The resulting equation is:

$$EHBF = (Vd \cdot K)/E$$

Since the clearance technique is favored over the other techniques it is appropriate to consider potential sources of error. First, in calculating extraction, two blood samples are required, arterial and hepatic venous. However, the hepatic vascular bed obtains its blood supply from two sources, the hepatic artery and portal vein. If a substance is extracted only by the liver its concentration in the hepatic artery and portal vein must be identical during an infusion. After a bolus dose the concentration of substance entering the liver will be composed of the hepatic arterial and portal venous blood concentrations. These two concentrations will not be identical by virtue of the transit time required for the arterial blood to traverse the intestinal circulation. The time period will also vary between animals. If the transit time is known then the concentration of test substance entering the liver can be calculated. A similar problem exists with the hepatic venous blood sample. This sample must be taken one transit time (time required for the blood to pass through the hepatic vascular bed) later than the hepatic arterial sample. These transit times will affect the calculated extraction greatly if the test substance has a very short half-life, e.g. indocyanine green. Furthermore, if extraction is assumed to be 100% and is not, then the total

calculated hepatic blood flow will be underestimated by the magnitude with which the extraction differs from unity.

If the test substance is extracted by splanchnic organs other than the liver, then the portal venous blood concentrations will be much less than the arterial concentrations. Without prior knowledge of the extrahepatic clearance, the calculated extraction will not reflect true hepatic extraction but will be equal to hepatic and extrahepatic extraction. This will also result in an incorrect estimation of hepatic blood flow. It is possible that a situation exists where the extrahepatic extraction compensates for the incomplete hepatic extraction. In this case the two extractions balance and a fortuitously correct blood flow is calculated (see Results).

Distribution must be achieved rapidly following a bolus dose. Sampling of arterial and hepatic venous blood should follow the initial distribution phase. The blood concentration-time curve should be described by a single exponential over the entire concentration range. No substance reported has been described by a single exponential. Thus, either distribution has not been achieved or extrahepatic uptake exists. Indocyanine green has been shown to give greater estimates of blood flow then

the electromagnetic flowmeter when a simple mixing chamber was used. The overestimation was reported to be due to inadequate mixing (85).

In many cases extraction of the test substance is assumed to be 100% thus circumventing the need for hepatic vein catheterization. Efficiency of the hepatocytes to extract the test substance is also assumed not to be affected by the presence of hepatic disease or other drugs administered concurrently. However, it is known that extraction efficiency of hepatocytes decreases in liver disease (53). Whether extraction decreases with concurrently administered drugs is not known.

Another potential source of error exists with the sampling of hepatic venous blood. It is assumed that a single hepatic vein sample is representative of the total hepatic venous outflow in healthy (79) and diseased livers (53). One study has demonstrated that in normal individuals a single hepatic venous blood sample represents the total outflow concentration (202). Data from other studies have also demonstrated that similar concentrations are obtained when blood is sampled simultaneously from two separate venous catheters (185). Conversely, studies have shown that the resulting concentrations analyzed may vary by 7% to 36% when blood is sampled simultaneously from two

catheters positioned in different parts of the liver (189, 202). The variability in concentrations may be due to placement of the catheter at various positions within the hepatic vein. The estimated hepatic blood flow calculated from samples obtained from the common hepatic vein gave 40% greater flow estimates than those which were obtained with catheters placed deep within the vein (175). Sapirstein and Reininger (175) suggested that resistance changes are produced which are important in determining the inflow to the catheterized area. This area may then become ischemic. The ischemia will result in an increased arteriovenous difference for the dye moving through that portion of the liver. Their results may be explained in terms of blood flow through the catheterized region of the liver. Since an obstruction to flow is present (i.e. catheter), blood flow through that region is reduced. The decreased flow increases the transit time of blood in that area, such that there is more time for the substrate to interact with the cell surface. The net result is an increase in uptake and an underestimation of hepatic blood flow. Variabilty in BSP extraction between lobes has also been reported (40, 51). Thus, hepatic vein catheterization may yield reliable information if some strict guidelines are followed. These are: position of the catheter tip should be deep in the hepatic vein but not in a wedged position; blood should be withdrawn freely; a volume of fluid be discarded before the

actual sample is obtained; and estimated hepatic blood flow should be based on the mean concentration of simultaneous samples from two or more hepatic veins (40, 79, 87).

Clinically, it is difficult to verify that extrahepatic uptake of the test substance does not exist. One approach used the arteriovenous difference across other organs demonstrating that it is essentially zero (19). However, at any one point in time the arteriovenous difference may be small but this does not disprove extrahepatic uptake since small differences are difficult to detect. Proof that extrahepatic uptake is negligible must come from hepatectomized animals. The test substance should not disappear from the blood in this preparation.

The elimination rate must also remain constant over the concentration range and sampling time period. The apparent elimination rate constant should not be different during repetitive admininstration of the test substance (e.g. at 40 minute intervals).

Thus, to use the clearance method for estimating hepatic blood flow properly requires proof of these requirements. The clearance method has never been critically evaluated by comparison with either the electromagnetic flowmeter or a direct timed collection of

hepatic venous blood. As well, the estimated flow must be shown to equal the measured flow when flow is varied over a wide range. These requirements have not been demonstrated in an animal model.

3. TEST SUBSTANCES

a. Indocyanine green

Indocyanine green (ICG), a tricarbocyanine dye, was introduced into clinical medicine by Fox, et al. (45, 46, 47), for measuring cardiac output. Chemically it is an anhydro-3,3,3',3'-tetramethyl-1,1'-di-(4-sulfobutyl)-4,5,4',5'-dibenzoindotricarbocyanine hydroxide sodium salt (Fig. 1) having a molecular weight of 775. The dye is neither strongly acidic nor strongly basic since it does not change the pH of triple distilled water. ICG is highly soluble in water but poorly soluble in saline. Once dissolved in water, salt may be added without precipitation of the dye (47).

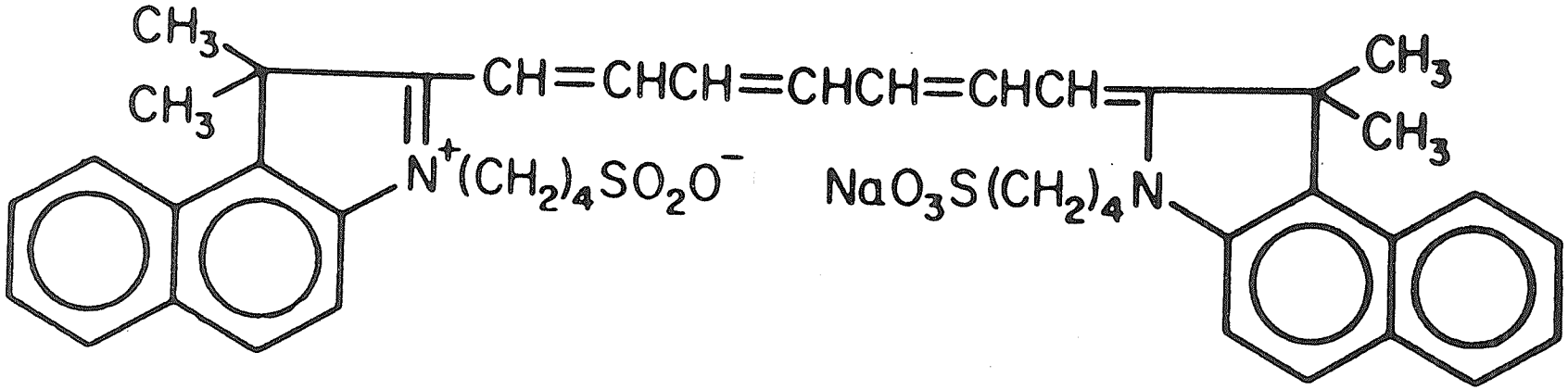
ICG may be quantitated using spectrophotometry since it obeys Beer-Lambert's Law over a suitable concentration range. The linear concentration range of ICG reconstituted in water is much smaller than that in plasma (105). When reconstituted in distilled water ICG yields two peaks. One

peak, identified as ICG, has an absorption maximum between 770 and 780 nm (3, 105, 178). A second peak, thought to be a degradation product, has an absorption maximum between 720 and 700 nm (146). ICG degrades rapidly in water. The peak between 720 and 700 nm increases while the peak between 770 and 780 nm decreases (5, 105). Degradation of less concentrated solutions is faster than that of the more concentrated solutions, possibly due to the fact that ICG forms aggregates at higher concentrations (178). Deterioration is slower when the dye solution is kept in the dark (49). Concentrated solutions are deemed stable for up to 2 days after preparation (45).

Addition of protein to the aqueous solution increases stability of ICG (49). This also leads to a shift in the absorption maximum into the region between 790 and 805 nm (3, 49, 125). The shift in absorption peak compared to that of ICG in water is suggested to be evidence for protein binding (67). Protein binding of ICG has also been shown through electrophoretic and ultracentrifugation studies (45). The linear concentration range for ICG in plasma was shown to be up to 19.4 nmol/ml. A slight deviation occurs at concentrations between 19.4 nmol/ml to 38.7 nmol/ml (105). The greater linearity in optical density and ICG concentration in plasma as compared to water may be due to the protein binding rather than

Fig. 1. Chemical structure of indocyanine green.

$C_{43}H_{47}N_2NaO_6S_2$ Molecular Weight 774.96



aggregate formation (105).

ICG is rapidly and almost entirely bound to plasma proteins after intravenous (i.v.) administration (3). Thus, it is distributed within the plasma protein compartment (199). This has been substantiated by comparing the initial distribution of ICG with that of ^{131}I-albumin clinically (25) and experimentally (199). It is not known to which protein ICG is preferentially bound. Fox and Wood (47) suggested that it is mainly bound to plasma albumin. Baker (3) noted that 90% of the dye in dogs and 80% in humans is bound to globulins, probably alpha$_1$-lipoprotein. In very low concentrations, Kamisaka, et al. (88), showed that it binds preferentially to beta-lipoprotein. It may be that the globulins have a limited number of high affinity binding sites for ICG. Once these sites are saturated ICG will bind to albumin. Thus, at low concentrations it may bind to globulins while at higher concentrations it may bind to both globulins and albumin.

ICG is thought to be eliminated from the blood solely by the liver. After i.v. administration ICG is completely recovered from bile in an unaltered form (93, 199). It does not lend itself to enterohepatic circulation since it was not found in the bile after a 59.4 µmol dose was

administered into the duodenum of one dog (199).

The LD_{50} of ICG in mice is between 77.4-103.2 μmol/kg, 64.5-90.3 μmol/kg in rats and between 64.5-103.2 μmol/kg in rabbits (103). Toxic effects of ICG have not been reported in humans after receiving bolus doses of up to 6.5 μmol/kg (126) and oxygen consumption by the perfused liver was not changed after the administration of ICG (171). ICG has been reported to inhibit mitochondrial oxygen consumption. The decreased oxygen consumption was suggested to be the result of a decrease in substrate penetration in the presence of ICG (106).

Studies have demonstrated that uptake of ICG by kidney or lung tissues is negligible (25, 92, 125, 152, 158) and urinary excretion does not ocurr (19, 25, 80, 92, 125, 152, 199). It is not detected in spinal fluid (92, 152) but small concentrations are detected in hepatic lymph (80). Studies which attempt to disprove extrahepatic uptake commonly sample arterio-venous blood randomly. Although data available suggest no extrahepatic uptake, small differences are difficult to detect due to the accuracy and precision of the analytical assay. In hepatectomized dogs, ICG was found to have an apparent elimination rate of 0.0016/min (80). This amounted to 2% of the normal apparent elimination rate which for dogs is 0.088/min (80, 199).

The extrahepatic apparent elimination rate may be somewhat greater than that calculated in the previous study. In dogs, after a 25.8 μmol bolus dose, there was an exponential decline in plasma ICG concentrations. During this period the hepatic venous ICG concentrations were greater than the arterial concentrations (189). Hepatic venous blood was sampled via hepatic vein catheterization. Although the authors did not comment on this observation, it may be due to significant extrahepatic uptake at a time when the liver becomes saturated with ICG. The greater hepatic venous plasma ICG concentrations than the arterial concentrations may be due to the release of ICG by the hepatocytes back into the plasma or the errors involved in sampling hepatic venous blood.

Binding sites for ICG as well as for BSP have been demonstrated is isolated hepatic cell plasma membranes (33). These sites are thought to play an important role in carrier-mediated transport of the dyes. Some substances have been shown to compete with ICG for the transport mechanism. BSP, bilirubin and rifamycin have been shown to increase the Km for ICG (145, 177). In man, jaundice has been shown to decrease the elimination rate of ICG (81). Competition of these substances for the available binding sites on the hepatocyte membrane may be one possible explanation. Within the hepatocyte ICG is bound to

ligandin (127, 159). This binding protein was suggested to serve as a storage site prior to biliary excretion (95, 199).

Since ICG is eliminated exclusively by the liver with negligible extrahepatic uptake, clearance of this dye was used for some 25 years in estimating hepatic blood flow both clinically and experimentally (4, 19, 199). ICG is assumed to be highly extracted by the liver and commonly administered as a 0.65 or 1.3 μmol/kg bolus or 0.65 μmol/min infusion (19). After a bolus dose administration, blood is usually sampled at 3 minute intervals for up to 21 minutes. The apparent elimination rate, which is commonly assumed to be linear for the plasma concentration-time relationship, is used along with the calculated apparent volume of distribution to estimate hepatic blood flow. There are two problems using this simplistic approach. First, many reports have indicated that ICG follows dose dependent kinetics. As the administered bolus dose is increased the apparent elimination rate constant decreases (81, 95, 189, 190). The resulting plasma concentration-time relationship is not expressed by a single exponential but rather by a multi-exponential equation. This is true if blood is sampled for a sufficient period of time and the analytical method is sensitive to detect small concentrations. Consider, in the

simplest terms, that the resulting plasma concentration-time relationship for ICG following a bolus dose may be described by a biexponential equation. The initial linear portion (or alpha phase) in the equation represents distribution (i.e. distribution within the vascular system and/or extrahepatic sites) as well as elimination. The calculated hepatic blood flow using the initial linear portion (alpha phase) would be expected to overestimate true hepatic blood flow if extrahepatic uptake exists. The beta portion of the biexponential equation, which reflects some redistribution but mostly elimination, would be expected to give a more reliable result for blood flow. It should be noted that there is no reason to assume that a biexponential equation describes the data. It is used here only for simplicity (for a review of compartmental analysis see ref #50). Many elaborate kinetic models have assumed as many as four compartments (190, 193).

The second problem is in the assumption that ICG is completely removed by the liver during a single pass. Many reports, both early and recent, clearly show that extraction of ICG is not 100% in either humans or animals. Caesar, et al. (19), have shown that mean extraction in humans following a 0.65 μmol/kg bolus dose was 68%. Extraction decreased to a mean of 62% in cirrhotic livers.

During the 0.65 μmol/min infusion, extraction was 62% in normal and 49% in cirrhotic livers. It is interesting that during their infusion study the extraction was similar to that of the bolus dose despite a much lower resulting plasma ICG concentration. A greater extraction would have been predicted since at the lower plasma concentrations a greater apparent elimination rate would be expected if ICG follows dose-dependent kinetics. Similarly, others have shown that extraction of ICG following a 0.65 μmol/kg bolus dose was highly variable with various types of liver diseases, range 1-100% (80, 146). Many other studies in man demonstrate that extraction of ICG by the liver is not 100% (24, 30, 200, 202). In animals, extraction is much lower than that reported for humans. Mean extraction in dogs is approximately 15% and decreases slightly with repetitive administrations (4). During an infusion of 6.45 nmol/kg body weight/min, extraction in cats ranged from 20% to 33% (102). In rats extraction is 7.5% and 4.3% following bolus doses of 1.3 μmol/kg and 6.5 μmol/kg, respectively (83).

As discussed previously, to avoid sampling hepatic venous blood, steady-state must be achieved during an infusion of ICG in order to estimate hepatic blood flow. Although studies have concluded that the plasma concentration-time relationship demonstrates steady-state

(19, 34, 124, 171), there is a definite increase in both arterial and hepatic venous concentrations. This demonstrates that steady state is not achieved even when very low amounts of ICG are infused. Furthermore, extraction during this time cannot be 100% if the plasma concentrations are increasing.

Despite the fact that the available data indicate a potential error in the use of ICG to estimate hepatic blood flow, poorly designed studies are available which attempt to support the use of ICG clearance data to estimate hepatic blood flow. Almost all of these have compared the estimated flow obtained with ICG to that of other substances. Such reasoning results in a circular argument. For example, in one study mean estimated hepatic blood flow using ICG clearance data was found to be 12% greater than that obtained using BSP clearance data (4). It is known that BSP is cleared by extrahepatic sites (28). Therefore, BSP estimated flow is greater than true hepatic blood flow. ICG clearance data would then be expected to result in an even greater overestimate. Shoemaker (185) found a 10% discrepancy between the BSP estimated flow and the electromagnetic flowmeter using a canine perfused liver preparation. Similar results were obtained by Drapanas (38). Earlier, Selkurt (180) showed that BSP estimated flow resulted in sightly higher values than the directly

measured flow. ICG estimated flow has also been compared with the indicator dilution technique using ^{131}I albumin (30) and ^{99m}Tc-diethyl-Ida (135). Hepatic blood flow estimated using ICG was similar to that of the other substances. Whether these substances give reliable estimates of flow is not known. Three 'different methods' for estimating hepatic blood flow were investigated by Pirttiaho, et al. (149), $^{99}Tc^{m}$-sulfur colloid, ICG and ^{133}Xe wash-out. A good correlation was found between ICG and $^{99}Tc^{m}$-sulfur colloid but a poor correlation was found between $^{99}Tc^{m}$-sulfur colloid and ^{133}Xe. These studies are good examples of circular reasoning. Again, it is not known which substance yields reliable flow measurements, the ^{133}Xe, $^{99}Tc_{m}$-sulfur colloid or ICG. Numerous other studies such as these exist in the literature (86, 185, 202). Nxumalo, et al. (140), compared the ICG clearance method against the electromagnetic flow method. As discussed earlier, the electromagnetic flow method gives reliable results if used properly. A canine septic shock model was used to alter hepatic blood flow. Their study showed a good correlation between the two methods at control flow (correcting for the hepatic extraction fraction). When blood flow was altered, due to septic shock, the ICG method underestimated the electromagnetic flow result by 20%. However, no mention was made concerning flow probe calibration. In a second study by

Nxumalo, et al. (139), dextran 40 was infused to induce a high cardiac output state, thus, increasing hepatic blood flow. In this study, ICG estimated flow consistently gave higher results than the electromagnetic flow recordings. Possible explanations included; a) dextran 40 may have decreased ICG extraction; b) dextran 40 blocked or competed with ICG uptake; or c) there was increased shunting of blood. A similar result was obtained by Jacobs, et al. (85), however, no explanation was offered.

ICG in plasma has conventionally been analyzed by the spectrophotometric (SPEC) method (19). Available data indicate that this organic anion is excreted into the bile unchanged (5, 19, 25). Recently a degradation product or metabolite of ICG has been reported in rabbits (156, 193). Plasma samples were analyzed after the administration of a 34.2 μmol/kg bolus dose by high pressure liquid chromatography (HPLC). The reported product has some light absorbing capacity at the same wavelength as ICG. The SPEC method was subsequently suggested to be nonspecific for the determination of ICG clearance, yielding erroneously low clearance values. The substance was not found to be excreted into the bile. Donn, et al. (37), substantiated this report by showing the existence of this substance in human plasma after the intravenous administration of a 0.6 μmol/kg bolus dose. Their reported

sensitivity of the SPEC method (65 pmol/ml) suggests that, at these very low concentrations, changes in background absorbance were being recorded as both ICG and the unknown substance. Sevensson, et al. (181), reported that the HPLC and SPEC methods yield identical estimates of ICG concentrations in plasma of humans at a clinically relevant dose (0.65 µmol/kg). The existence of this substance is somewhat controversial. It would appear that if the substance exists it is not present in sufficient concentrations to render the SPEC method unreliable in view of data from Nxumalo, et al. (139, 140).

b. Galactose

The use of galactose clearance data to estimate hepatic blood flow was first proposed by Tygstrup and Winkler (196). Renewed interest developed recently after the discovery of a more accurate and convenient method of galactose measurement in blood (74, 75, 76, 174).

Galactose is a water soluble, non-protein bound substrate which is highly extracted by the splanchnic circulation (75). Extraction in humans with normal liver function ranges between 88% to 94% during a single pass of the liver. Extraction during liver disease is often much lower (range 39%-95%; 75, 76, 196). Galactose is known to

be excreted in the urine and to be metabolized by erythrocytes (137, 195). Extrahepatic clearance is thought to be minimal and accounts for the difference in the incomplete hepatic extraction such that total body extraction is 100% (74).

Hepatic blood flow determined by the galactose clearance method was found to be similar to that obtained by the BSP clearance method (196). Since BSP is known to be cleared through extrahepatic sites, galactose and BSP will yield greater estimates of true hepatic blood flow. These data conflict with that of Henderson and Hanna (76). In their study, the galactose estimated flow was similar to that measured by the electromagnetic flowmeter in dogs (76). However, they examined only control flow and one infusion rate. Calibration and zeroing of the flowprobes were not discussed.

D. Statement of Problem

After reviewing the literature, many discrepancies were found to exist as to whether ICG and/or galactose are reliable indicators for the determination of hepatic blood

flow. There is a large amount of data demonstrating that a one compartment open pharmacokinetic model assuming a first order rate constant for hepatic uptake does not adequately describe the elimination process of ICG and that extraction of ICG is not 100%. Despite these data, ICG continues to be used to estimate hepatic blood flow and, at times, with the assumption that it is completely extracted (24, 43). The recent report suggesting that erroneously low clearance values are obtained using the SPEC method for ICG analysis (due to the presence of a metabolite or degradation product) has further clouded the reliability of this substance to estimate hepatic blood flow. Similar problems plague the use of galactose. Furthermore, the clearance method has never been critically evaluated experimentally. The method has never been compared to the electromagnetic flowmeter or a timed collection of hepatic venous blood in an animal model where hepatic blood flow was proposely altered over a wide range. Thus, it is not clear as to whether these test substances may reliably be used to estimate hepatic blood flow. Basic animal studies were needed to clarify the validity of the clearance method to estimate hepatic blood flow. In this study, the SPEC and HPLC methods for ICG analysis in plasma, liver tissue and bile were compared. Estimated hepatic plasma flow was calculated in various ways from the clearances after bolus injections of ICG. Estimated hepatic plasma flow was also

calculated from infusions of ICG and galactose in an experimental situation in anesthetized cats, where; a) repeated sampling does not deplete the animal's blood volume; b) true hepatic blood flow is measured directly and continuously throughout the experiments, and; c) hepatic blood flow was purposely altered. Clearance of ICG and galactose was also examined in hepatectomized cats.

METHODS

A. SURGICAL PREPARATION

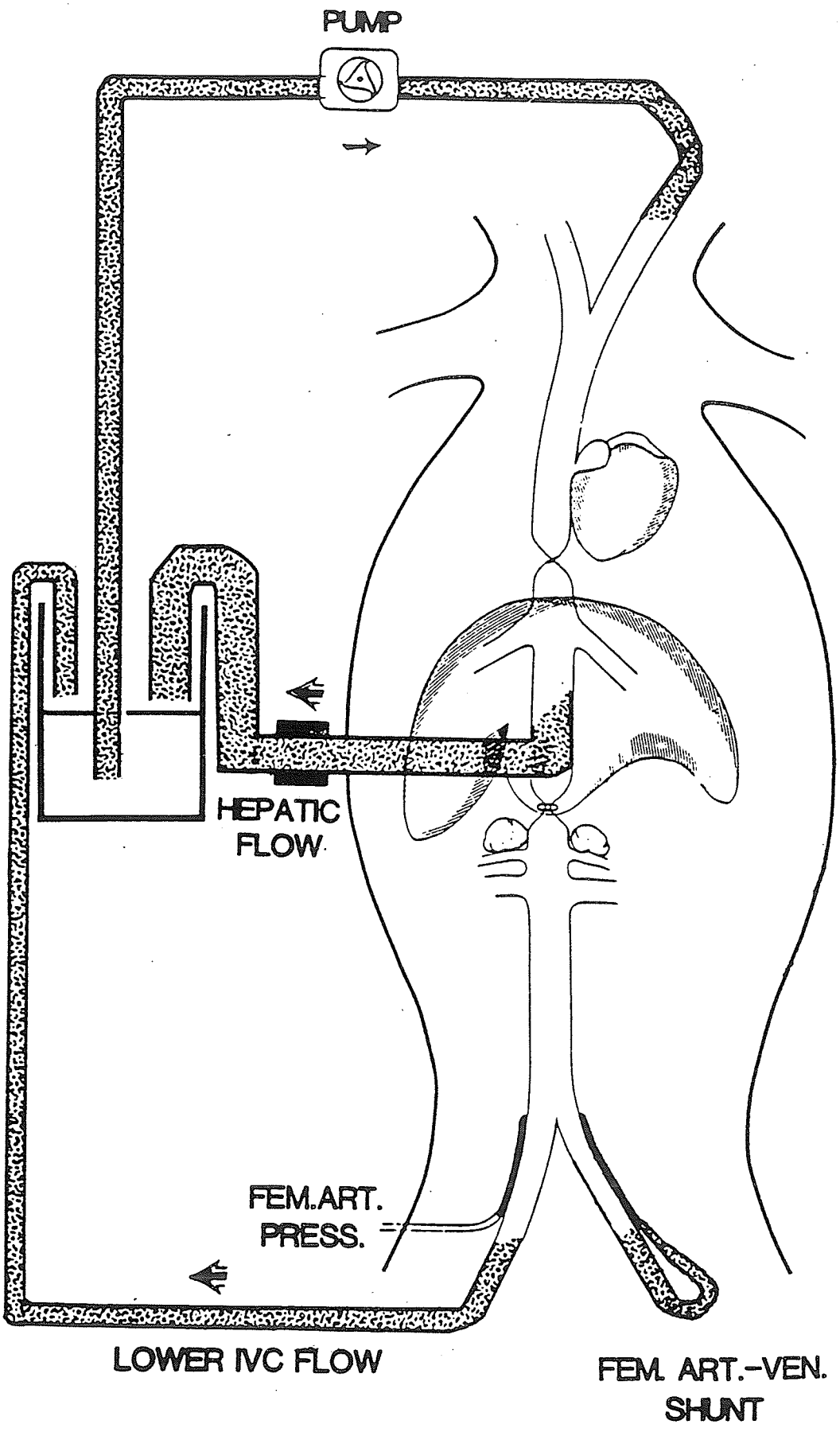
Cats, of either sex, weighing between 2.0-3.9 kg (2.8 ± 0.1 kg, mean ± SE) were anesthetized by intraperitoneal injection of pentobarbital sodium (120 mmol/kg). Supplementary doses of pentobarbital (24 mmol) were administered via a cannnula (polyethylene (PE) 90 tubing, i.d. 0.9 mm, Becton, Dickinson and Company) in the left cephalic vein when required to just suppress reflex swallowing movements in response to gentle traction of the tongue. Arterial pressure was recorded from the right femoral artery using a PE 240 tube with a tapered inlet, a Beckman 4-327-C pressure transducer and a Beckman type RM Dynograph. Calibration was performed periodically using a mercury manometer. Artificial respiration was maintained with a Harvard respirator and adjusted to maintain blood gases and pH within normal limits. Blood gases and pH were analyzed with a model IL 1302 pH/blood gas analyzer (Instrumentation Laboratory Inc.) throughout the experiments. Rectal temperature was maintained between 37 and 38^{o}C by a temperature controlled surgical table.

An extracorporeal long-circuit technique, modified from Greenway and Lautt (61), was used for direct measurement of hepatic blood flow and to allow repeated sampling of arterial and mixed hepatic venous blood without depletion of

the animal's blood volume. The preparation is shown in Fig. 2. It was set up in the following way to avoid occlusion or congestion of organs during the preparation. The left jugular and right femoral veins were prepared for later cannulation. The abdomen was opened through a mid-line incision and the inferior vena cava was isolated between the liver and the adrenal glands. The bile duct was cannulated (cannula size depended on the diameter of the common bile duct and varied between PE 90 and PE 160 tubing) and the cystic duct was ligated. A ligature was placed around the thoracic inferior vena cava through a small incision at the top of the diaphragm. The cat was then left for 30 min, while 80-100 ml of blood were taken from a donor cat into heparin (1000 units). The extracoporeal reservoir and associated tubing were primed with 50 ml of blood. The remaining blood was used to replenish the reservoir between each bolus dose of ICG such that the initial volume of the reservoir was constant. When an infusion of ICG or galactose was used, and in the hepatectomized series, the extracorporeal reservoir and associated tubing were primed with all of the donor cat blood. The reservoir was depleted as samples were taken throughout the experiments. Blood temperature was maintained at 37-38^{o}C by a water-bath surrounding the reservoir.

The left jugular vein was cannulated (Teflon, Medical

Fig. 2. Diagram of the preparation which allowed direct measurement of total hepatic blood flow and repeated blood sampling without depletion of the cat's blood volume.


PUMP
HEPATIC
FLOW
FEM.ART.
PRESS.
LOWER IVC FLOW
FEM. ART.-VEN.
SHUNT

Grade, i.d. 1.7 mm, Becton, Dickinson and Co., N.J.) and connected to the pump which returned blood from the reservoir to the animal. The right femoral vein was cannulated (Teflon, Medical Grade, i.d. 2.9 mm) and connected to a tube (Masterflex^{R} silicone tubing, size 16, Cole-Parmer Instrument Company, Chicago) which drained to the reservoir. The cat was given heparin (500 units intravenously). Extracorporeal flow was begun slowly to allow mixing of the donor blood in the animal. The inferior vena cava was occluded between the liver and the adrenal glands so that venous blood from the lower part of the animal drained from the femoral vein cannula to the reservoir, and was returned to the animal via the jugular vein cannula. A cannula was inserted into the inferior vena cava (Teflon, Medical Grade, i.d. 2.9 mm) above the point of occlusion so that its tip lay at the entry of the hepatic veins. This cannula was connected to the extracorporeal reservoir and the thoracic inferior vena cava was occluded. The level of the outflow pipe was set to maintain a pressure close to zero in the hepatic segment of the inferior vena cava. Total hepatic flow now passed through this tube and was measured by an extracorporeal probe of an electromagnetic flowmeter (Model FM501D, Carolina Medical Electronics Inc.). A short by-pass around the probe allowed repeated zero flow checks. The flowmeter was calibrated periodically by timing the collection of 10 ml of hepatic

venous effluent. Mixed hepatic venous blood samples were taken from the outflow tube when required. A femoral arteriovenous shunt (using PE 240 tubing with a tapered tip) was prepared to allow sampling of arterial blood directly with a needle and syringe without deadspace problems. In some experiments portal blood was sampled through a catheter introduced into the portal vein through a branch from the appendix.

A similar extracorporeal long-circuit technique was used for experiments where hepatectomy was performed. In these experiments, the spleen was removed and the portal vein was cannulated (teflon medical grade tubing, i.d. 2.9 mm, Becton, Dickinson and Company) via the splenic vein and drained to the reservoir. The portal vein was then ligated at the hilum of the liver. The diaphragmatic veins, hepatic artery and common bile duct were ligated. The liver was then removed. Portal venous blood samples were taken from the splenic vein outflow tube.

B. PREPARATION OF ICG AND GALACTOSE

Since ICG (Cardiogreen, Hynson, Westcott and Dunning, Inc.; mol. wt 775) degrades rapidly in distilled water (105, 156), it was freshly prepared for each experiment. Each

dose was reconstituted in distilled water after which bovine serum albumin was added (BSA, fraction V, Sigma Chemical Co., 100mg/ml). ICG may be stored for several weeks at 4°C without significant degradation if sufficient protein is present (105, 156).

D(+)Galactose (Sigma Chemical Co.) was freshly prepared for each experiment in distilled water.

C. EXPERIMENTAL SERIES

Bile was collected continuously in 10 min aliquots during experiments in which ICG was administered. Livers (and kidneys in some experiments) were excised upon completion of these experiments. Plasma, liver, kidney and bile samples were immediately refrigerated at 4°C and were analyzed within 48 h. ICG and galactose were administered into the brachial vein either as an intravenous infusion or bolus dose. Arterial and mixed hepatic venous blood samples were taken during bolus dose administrations prior to and at 3, 6, 9, 12, 16, 20, 25, 30 and 35 min after each dose unless otherwise stated. When an infusion was given, blood was sampled every 10 min until the experiments were completed. To allow for hepatic transit time, all hepatic venous samples were taken 15 seconds after the arterial

blood was sampled. This time was based on an average total hepatic flow of 120 ml/min/100 g liver and a hepatic blood volume of 30 ml/100 g liver (66).

Eight series of experiments were carried out in the following way:

Series 1 (N = 5), hepatic blood flow was not altered. Three ICG bolus doses were administered at 40 min intervals. Each 1.0 ml dose contained 1.3 μmol ICG/kg body weight and was administered over approximately 3 seconds. This set of experiments was terminated 6 hours after the first dose of ICG.

Series 2 (N = 5), hepatic blood flow was maintained at the control flow rate for 40 min, increased to 150% control for 40 min by infusing blood into the animal from the reservoir, decreased to 50% control for 40 min by draining blood from the animal into the reservoir and finally returned to the control flow rate for an additional 120 min. Four bolus doses of ICG were given (1.3 μmol/kg body weight, 1.0 ml/dose) at t=0, 40, 80 and 200 min.

Series 3, involved investigating extrahepatic clearance of ICG (N = 5) and galactose (N = 3) in hepatectomized cats. ICG (1.3 μmol/kg body weight) and galacotse (1250 μmol/kg

body weight) were administered simultaneously as single bolus doses. Arterial and portal venous blood samples were taken at 0, 3, 6, 9, 12, 16, 20, 25, 30, 35, 40, 50, 60, 75, 90, 105, 120 and 135 min. Portal venous blood was sampled 15 sec after the arterial blood sample was obtained.

Series 4 (N = 4), ICG was administered as a constant infusion of 3.2 nmol/min/kg body weight for 150 min followed by 6.4 nmol/min/kg body weight for an additional 90 min. Hepatic blood flow was maintained at the control rate throughout these experiments.

Series 5 (N = 5), ICG was administered as a constant infusion of 6.4 nmol/min/kg body weight for 210 min. During the first 90 min, hepatic blood flow was maintained at the control rate. During the next 60 min period, hepatic blood flow was increased to 150% control and then reduced to 50% control for an additional 60 min.

Series 6 (N = 6), hepatic blood flow was not altered while 4 doses of galactose (5, 10, 15 and 20 μmol/kg body weight/min) were infused for 90 min per dose.

Series 7 (N = 4), galactose was infused at a constant rate (10 μmol/kg body weight/min) while hepatic blood flow was varied. After an 80 min control period at the initial

flow, hepatic blood flow was increased to 150% of control for an additional 60 min, reduced to 50% of control for another 60 min and then returned to the control flow rate for a further 60-90 min.

Series 8 (N = 8), hepatic blood flow was varied in the same way as in the seventh series. Galactose was infused at 25 μmol/kg body weight/min throughout these experiments.

D. INDOCYANINE GREEN AND GALACTOSE ANALYSIS

Plasma, liver and bile samples were analyzed by a high pressure liquid chromatography (HPLC) method modified from Rappaport and Thiessen (156). A chromatography pump (Waters, model 6000A), automatic sampler (Waters, WISP model 710B), variable wavelength absorbance detector (Hitachi, model 100-40) and a Fisher recorder (series 500) were used. Separation was performed with a RP-18 precolumn (Brownlee Labs) and a 3.9 mm (ID) X 30 cm C18 reverse-phase uBondpak column (10 μm particle size, Waters Scientific). The mobile phase was 47 parts acetonitrile (Fisher, HPLC grade), 3 parts methanol (Fisher) and 50 parts 0.05M pH 6 KH_2PO_4-Na_2HPO_4 buffer. Flow rate of the mobile phase was 1.7 ml/min.

Plasma was separated from arterial and mixed hepatic venous blood by centrifugation for 3 min (Fisher micro-centrifuge model 235A). An aliquot of the resulting supernatant was analyzed by the spectrophotometric (SPEC) method (19). This aliquot was diluted with 2 parts distilled water and analyzed using a Beckman DU-8 spectrophotometer at 803 nm. An equal volume of acetonitrile/methanol (47/3) was added to the remaining plasma, mixed for 30 sec (vortex-type mixer) and recentrifuged. An aliquot of the resulting supernatant (50 µl) was injected directly. ICG standards for the SPEC and HPLC methods were prepared in an identical manner in plasma obtained from donor cats. The optimal wavelength for ICG detection in acetonitrile/methanol was 783 nm. Detection of ICG by HPLC was performed at this wavelength for all samples.

Bile samples were diluted with acetonitrile/methanol, mixed for 30 sec and centrifuged for 3 min. Bile standards were prepared from control samples obtained before ICG administration. Both the prepared and test samples were processed identically. The concentration of ICG collected over the 10 min period in bile was corrected for the lag time due to the volume of the cannula. Samples were analyzed by both the SPEC and HPLC methods at 783 nm.

Upon completion of experiments, livers were excised, weighed and ICG content determined. Extraction of ICG from liver tissue was done by a modification of the method of Paumgartner, et al. (144). Liver lobes were homogenized using a Polytron (Brinkman Instruments; power setting 5 for approximately 5 min) after the addition of an equal volume of distilled water. BSA (0.5 ml, 100mg/ml) was added to 1.0 ml of the homogenate to simulate the volume change when standards were prepared. Liver proteins were precipitated with 8.5 ml acetonitrile/methanol (47/3), mixed for 30 sec and centrifuged for 10 min at 9000 x g. Standards were prepared with livers from donor cats by adding ICG in albumin solution to the liver homogenates. The remainder of the method was identical to that of the test samples. Samples were analyzed by both the SPEC and HPLC methods at 783 nm. ICG concentration in each liver lobe was measured to determine the uniformity of uptake. In some cats the kidneys were extracted and the ICG content determined in an identical manner to that of the liver tissue.

The galactose concentrations in whole blood samples from the femoral artery, portal vein and hepatic vein (25 µl) were analyzed within 5 min of collection using a Model 27 YSI Instrument (Yellow Springs Instruments, Ohio). This instrument incorporates a galactose oxidase membrane in a temperature controlled chamber. Background levels were very

low in the absence of infused galactose and few substances appear to interfere with the measurements (174). Direct readings of blood galactose concentration could be obtained over the range 50-10000 μM, with a coefficient of variation less than 5%.

E. PREPARATION AND ADMINISTRATION OF LABELLED BSA

The procedure used for labelling BSA with ^{125}I was modified from Katz and Bonorris (90) and Rosa, et al. (172). A solution (12 ml) of 2.5 x 10^{-4}M NaI in 0.9% NaCl was made from which 0.5 ml was added to the cathode. To the remainder (11.5 ml), 1.15 g BSA was added. The anode was filled with 5.0 ml of the BSA containing solution to which 0.5 mCi ^{125}I was added. Current was set at 0.4 mA for 30 min. After this time the labelled BSA and free ^{125}I were separated by passing the contents of the anode through a G25 fine Sephadex column (Pharmacia) which was conditioned the previous day by passing 1 ml BSA (10 mg/ml) and eluting with 0.9% NaCl. The labelled BSA and free ^{125}I eluant were washed through with 0.9 %NaCl and collected into approximately 30 1.5 ml fractions. The labelled BSA eluted within the first 10 fractions while the free ^{125}I eluted between fractions 20-30. The labelled BSA fractions were then dialyzed against 2.5 x 10^{-4}M NaI in 0.9% NaCl solution

until the dialysate had negligible counts.

Approximately 10^6 counts per minute (cpm) were injected into the animals. Blood volume was determined by taking 1.0 ml blood samples at 10 minute intervals for 30 min. An initial blood volume was then obtained by extrapolating the resulting log cpm-time curve to zero minutes by linear regression analysis. The volume of distribution of ^{125}I BSA at this time was taken as the circulating blood volume of the animal plus that of the reservoir and tubing.

F. STATISTICAL ANALYSIS

Data are presented as means $\pm$ S.E. Initial polyexponential parameter estimates were obtained using ESTRIP (17). Student's t-test for paired data, or blocked analysis of variance (ANOVA) with multiple comparisons by Duncan's multiple range test were used to determine significant differences. Standard curves for the assays were determined by linear regression using the least squares method and inter-assay variation was expressed as coefficient of variation (188).

RESULTS

A. HPLC ANALYSIS OF ICG

Wavelength scans of ICG in plasma and that extracted from plasma with acetonitrile/methanol (47/3) were examined. Results from the SPEC analysis (fig. 3) indicated that peak absorbance of ICG in plasma was at 803 nm. Peak absorbance of ICG extracted with acetonitrile/methanol was at 783 nm by the SPEC (fig. 4) and HPLC (fig. 5) methods. The addition of 6% methanol to acetonitrile was found to increase the stability of ICG (fig. 6; see ref #156).

HPLC chromatograms of plasma samples obtained 6 min after each ICG bolus dose (first series) together with the 12.9 nmol/ml plasma standard are shown in fig. 7. There were no detectable peaks in blank plasma samples. The 12.9 nmol/ml ICG plasma standard yielded two peaks, as did all arterial and mixed hepatic venous plasma samples. One peak was identified as the ICG parent compound having a retention time of 6 min. The other peak, eluting with a retention time of 4 min, was not identified. Peak absorbance of the unknown substance in the extraction solvent was at 780 nm. There was essentially no absorbance at 290 nm (fig. 5). After successive ICG bolus doses, peak height of the unknown substance increased (fig. 7). The increase in optical density of the unknown substance with

Fig. 3. Spectrophotometric wavelength scan of indocyanine green in plasma.

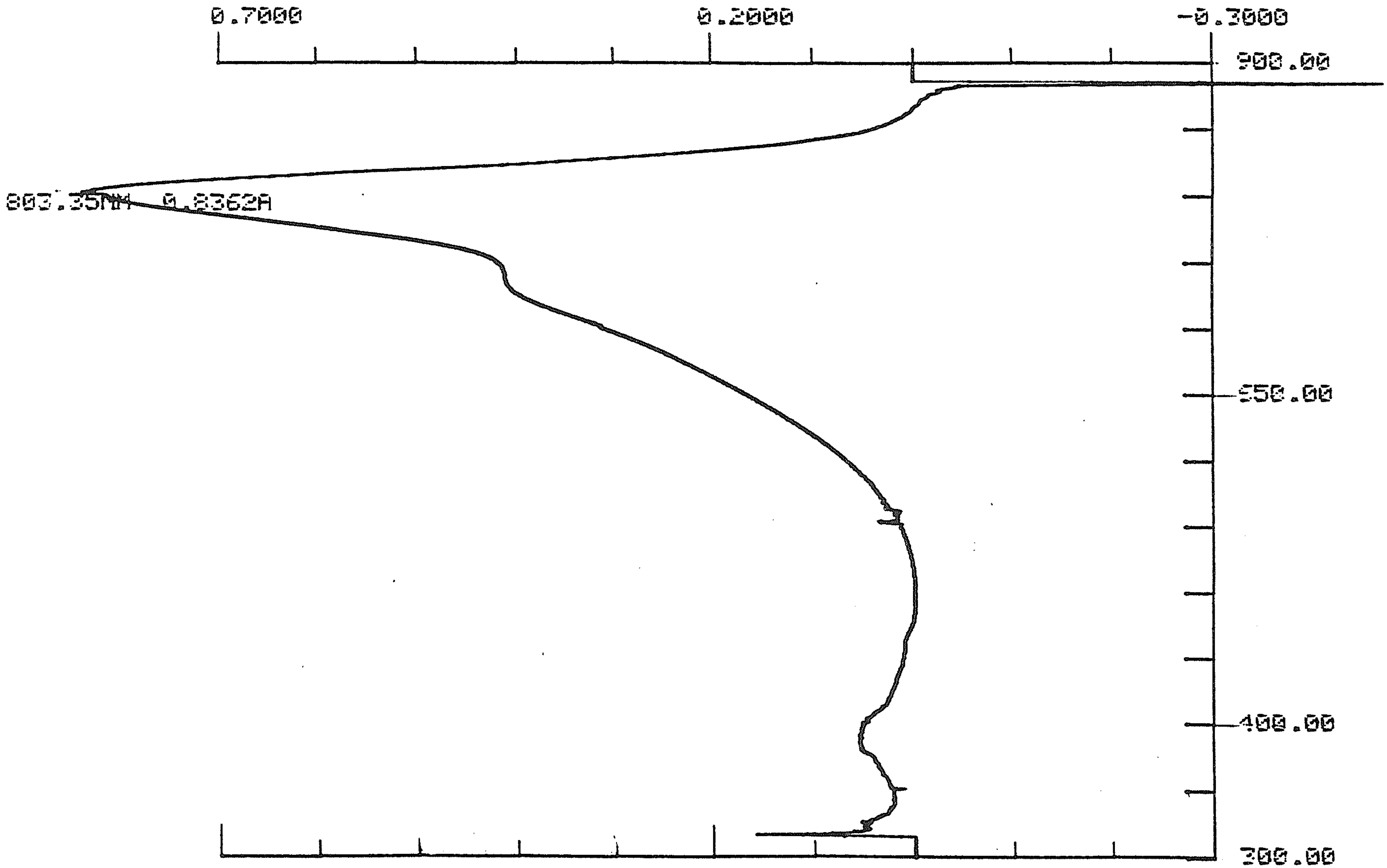

0.7000
0.2000
-0.3000
900.00
803.35NM 0.8362A
400.00
300.00

Fig. 4. Spectrophotometric wavelength scan of indocyanine green in acetonitrile/methanol (47/3).

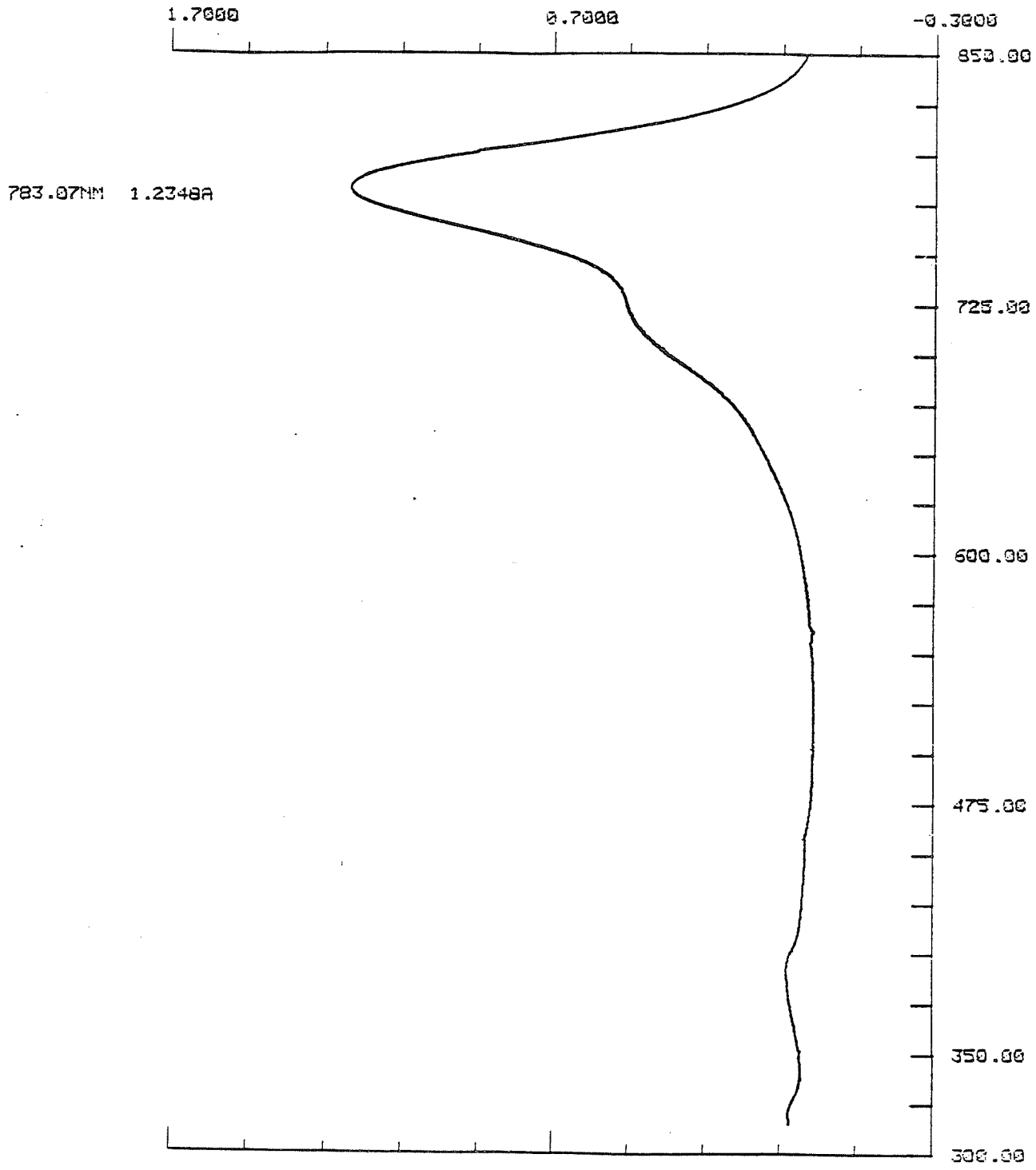

1.7000
0.7000
-0.3000
850.00
725.00
600.00
475.00
350.00
300.00
783.07NM 1.2348A

Fig. 5. High pressure liquid chromatographic wavelength scan of indocyanine green in acetonitrile/methanol (47/3). ● - ICG, O - unknown

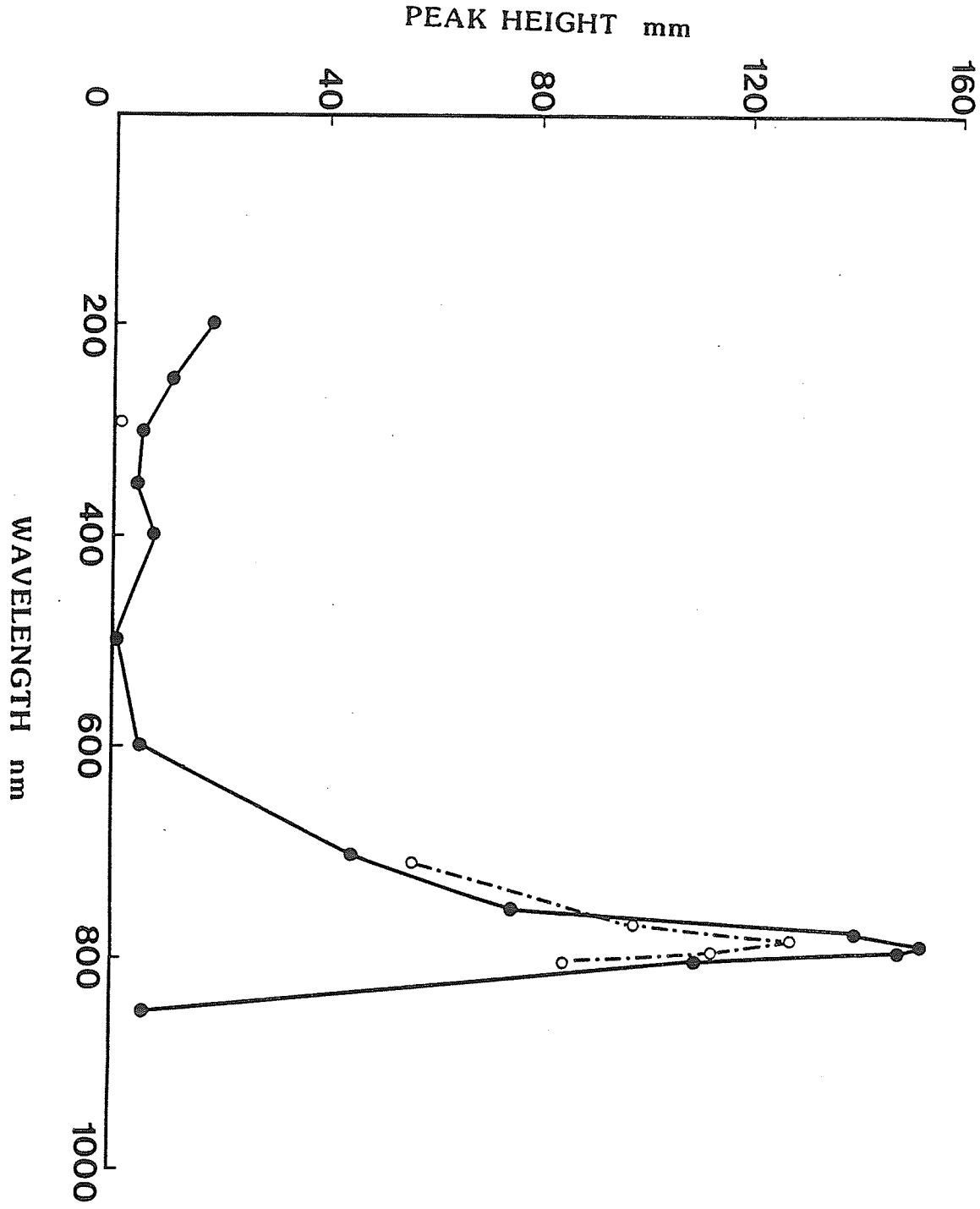

PEAK HEIGHT mm
0
40
80
120
160
200
400
600
800
1000
WAVELENGTH nm

Fig. 6. Stability of indocyanine green in acetonitrile/methanol (47/3). ● - indocyanine green, ○ - unknown substance. Mean ± SE, N = 3.

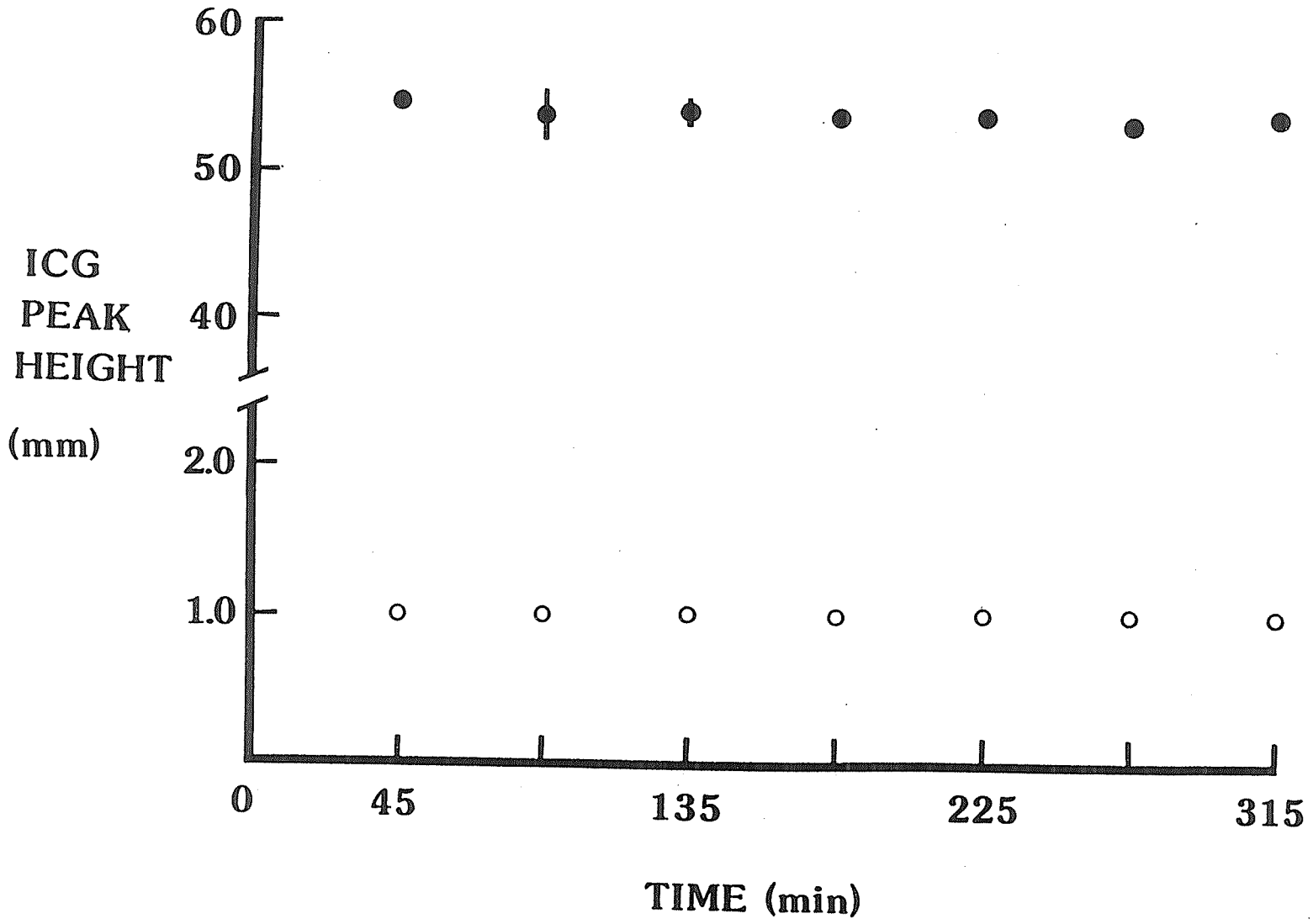
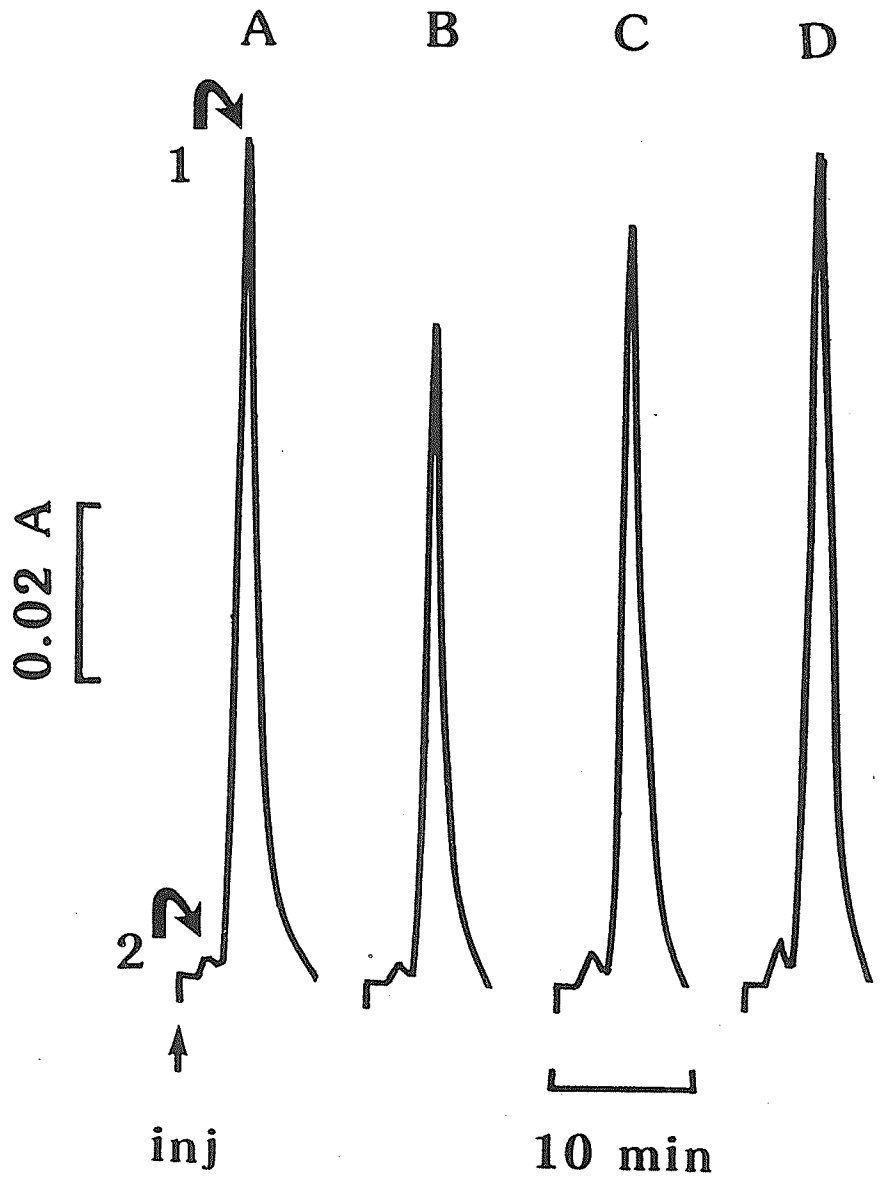

60
50
40
ICG
PEAK
HEIGHT
(mm)
2.0
1.0
0
45
135
225
315
TIME (min)

Fig. 7. High pressure liquid chromatograms obtained for: A - 12.9 nmol/ml plasma standard; B - 6 minute arterial plasma sample (first dose); C - 6 minute arterial plasma sample (second dose); D - 6 minute arterial plasma sample (third dose). 1 - represents indocyanine green, 2 - represents the unknown substance.


A
B
C
D
1
2
0.02 A
inj
10 min

successive doses resulted in slightly greater apparent ICG concentrations by the SPEC method (fig. 8). HPLC chromatograms of bile and liver samples were qualitatively similar to those of the plasma samples. Results from the chromatograms and retention times shown were obtained using a 30 cm x 2 mm i.d. column packed in our laboratory with the same packing as described previously (see Methods). Flow rate was set at 0.5 ml/min. Results using both columns were qualitatively consistent, the difference being in the retention times. Retention times using the column described previously (Methods) were reduced.

B. COMPARISON OF ANALYTICAL METHODS

The variability of measurements of ICG added to plasma, liver tissue and bile in various concentrations is shown in Table 1. ICG concentrations in plasma and low concentrations in bile (<15μM) determined by the HPLC and SPEC methods were not significantly different (figs. 8 and 9). However, the HPLC method was more sensitive than the SPEC method. The lower limit of detection was 0.1 μM for the HPLC method using a 25 μl sample size and 1.3 μM for the SPEC method using a 200 μl sample size. Standard curves for plasma, liver tissue and bile in the concentration range analyzed were linear (r^2=0.99) for both

Fig. 8. Comparison of plasma indocyanine green concentrations using the high-pressusre liquid chromatographic (HPLC) and spectrophotometric (SPEC) methods. The line of identity bisects the two axes. ● - first dose, □ - second dose, ■ - third dose.

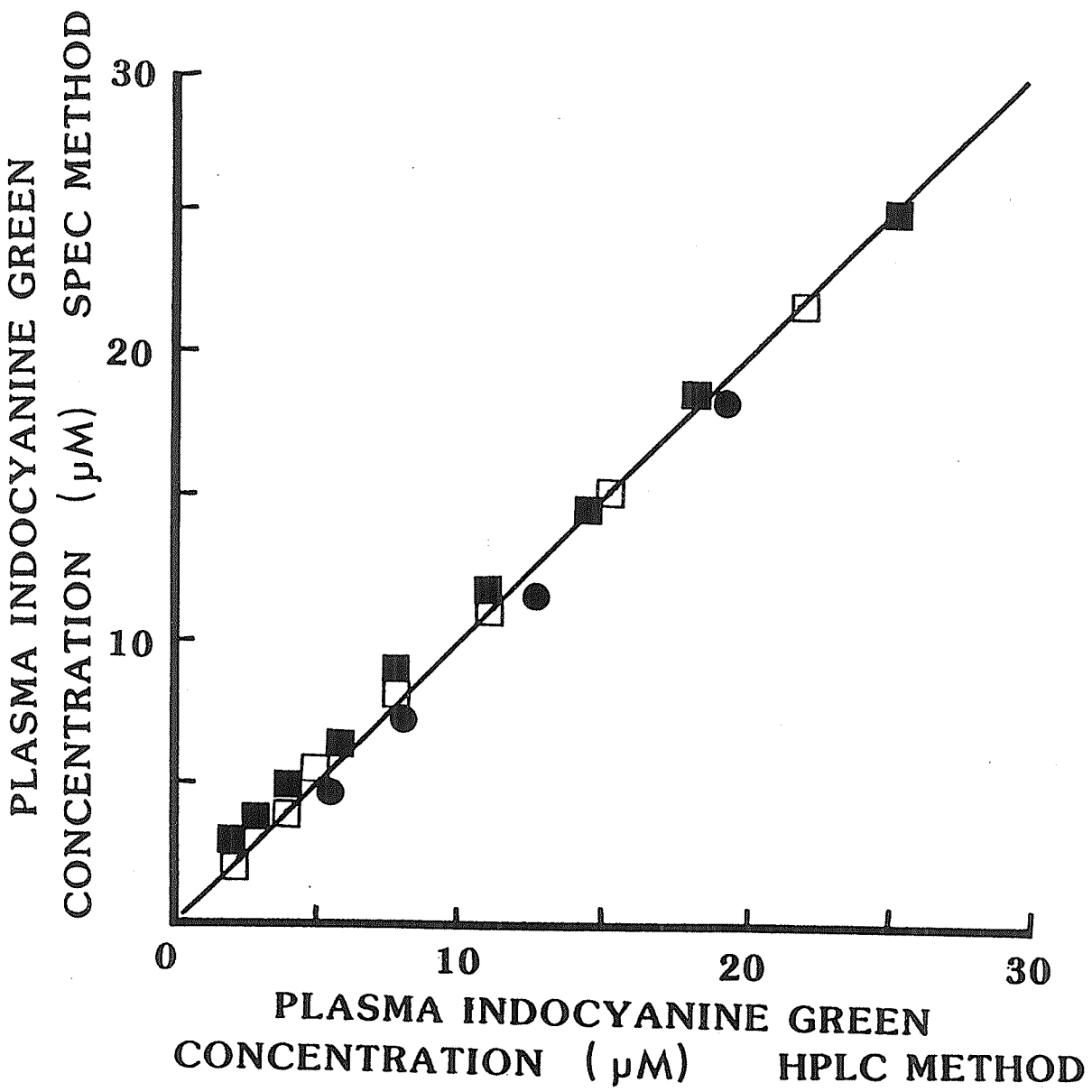

PLASMA INDOCYANINE GREEN
CONCENTRATION (μM) SPEC METHOD
30
20
10
0
10
20
30
PLASMA INDOCYANINE GREEN
CONCENTRATION (μM) HPLC METHOD

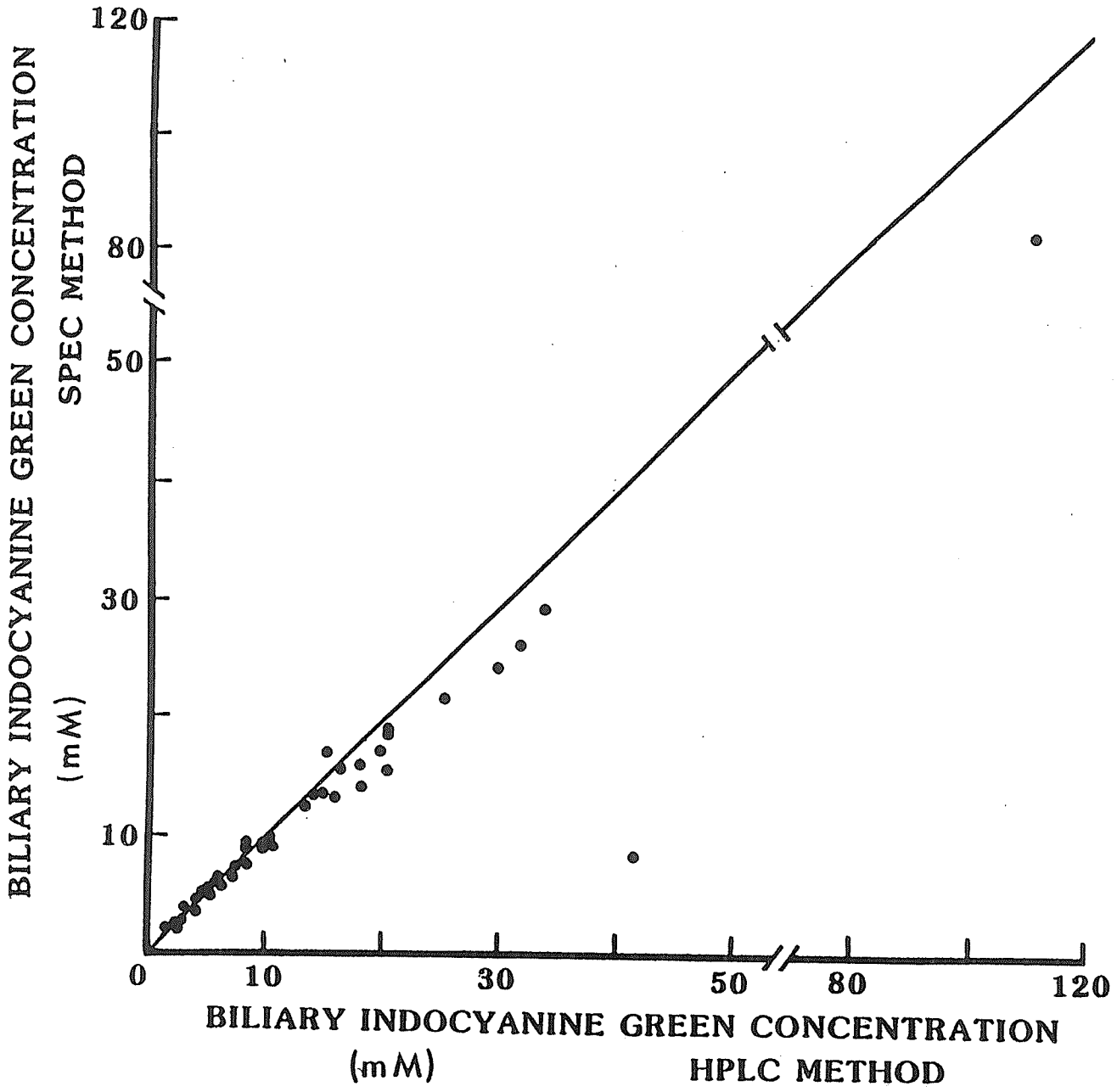
Table 1. Comparison of high-pressure liquid chromatography (HPLC) and spectrophotometric (SPEC) methods for estimation of ICG in plasma, bile (N = 5 at each concentration) and liver tissue (N = 10 at each concentration).

	Concentrations				
Sample	Prepared	Observed			
		HPLC		SPEC	
		Mean	CV	Mean	CV
PLASMA μM	51.6	51.5	0.8	47.0	6.2
	25.8	25.8	4.2	26.6	4.7
	12.9	12.8	3.7	13.4	2.1
	6.5	6.4	2.6	6.6	3.7
	1.3	1.2	10.1	0.7	23.3
	0.7	0.5	7.7	Not detectable	
BILE μM	51.6	51.8	0.7	15.9*	3.0
	25.8	25.1	3.3	15.8*	4.4
	12.9	12.8	2.0	12.7	0.9
	6.5	6.5	2.5	6.9	3.3
	1.3	1.2	27.1	1.4	42.6
LIVER nmol/g	103.2	103.0	1.0	102.6	1.0
	51.6	51.0	2.2	52.0	3.1
	25.8	25.6	5.1	26.0	3.3

Data are presented as mean and coefficient of variation (CV).

* $P<0.05$ for comparison between methods.

Fig. 9. Comparison of biliary indocyanine green concentrations using the high-pressure liquid chromatographic (HPLC) and spectrophotometric methods (SPEC). The line of identity bisects the two axes.


BILIARY INDOCYANINE GREEN CONCENTRATION
SPEC METHOD
(mM)
120
80
50
30
10
0
10
30
50
80
120
BILIARY INDOCYANINE GREEN CONCENTRATION
(mM)
HPLC METHOD

methods except for bile samples at concentrations above 15 uM in the SPEC method (Table 1). All test samples were analyzed within the linear range of the optical density-concentration relationship.

The amount of ICG in liver samples taken at the end of the first study was not significantly different between the two analytical methods and the liver contained 25 ± 5% of the total dose administered. ICG was uniformly distributed in all lobes of the liver (Table 2). The total amount of ICG in all bile samples accounted for 72 ± 6% of the administered dose. Thus 97% of the administered ICG was recovered in the bile and liver at the end of these experiments which were terminated 6 h after the first bolus dose.

C. ICG BOLUS DOSE ADMINISTRATIONS

In the first series of experiments, mean arterial pressures were 110 ± 6, 117 ± 6 and 123 ± 5 mm Hg, and hepatic blood flows were 148 ± 17, 131 ± 15 and 125 ± 15 ml/min per 100g liver at each of the three doses of ICG. Mean liver weight was 21.2 ± 1.3 g/kg body weight. Mean hematocrit was 38 ± 3% and did not change by more than 1% throughout each experiment.

Table 2. Comparison of high-pressure liquid chromatographic (HPLC) and spectrophotometric (SPEC) methods for the estimation of ICG concentrations in liver lobes. Mean ± S.E. for 5 cats.

LOBE	ICG CONCENTRATION (nmol/g tissue)	
	HPLC	SPEC
Right Lateral	50.0 ± 13.4	44.6 ± 11.6
Left Lateral	48.2 ± 11.8	47.8 ± 12.2
Right Medial	50.2 ± 11.8	48.2 ± 11.8
Left Medial	45.4 ± 10.6	44.8 ± 10.8
Caudate	48.0 ± 9.6	47.6 ± 10.4

Arterial pressures were 117 ± 4, 142 ± 3, 92 ± 14 and 117 ± 13 mm Hg and hepatic blood flows were 87 ± 7, 128 ± 10, 45 ± 3 and 86 ± 7 ml/min per 100g liver at each of the four doses of ICG for the second series, respectively. Mean liver weight was 22.1 ± 1.5 g/kg body weight. Mean hematocrit was 32 ± 1% throughout the experiments.

Initial, intermediate and final values for arterial and hepatic venous blood gases (pO_2 and pCO_2) and pH in all series of experiments are shown in Table 3. Blood gases and pH remained constant throughout all experiments.

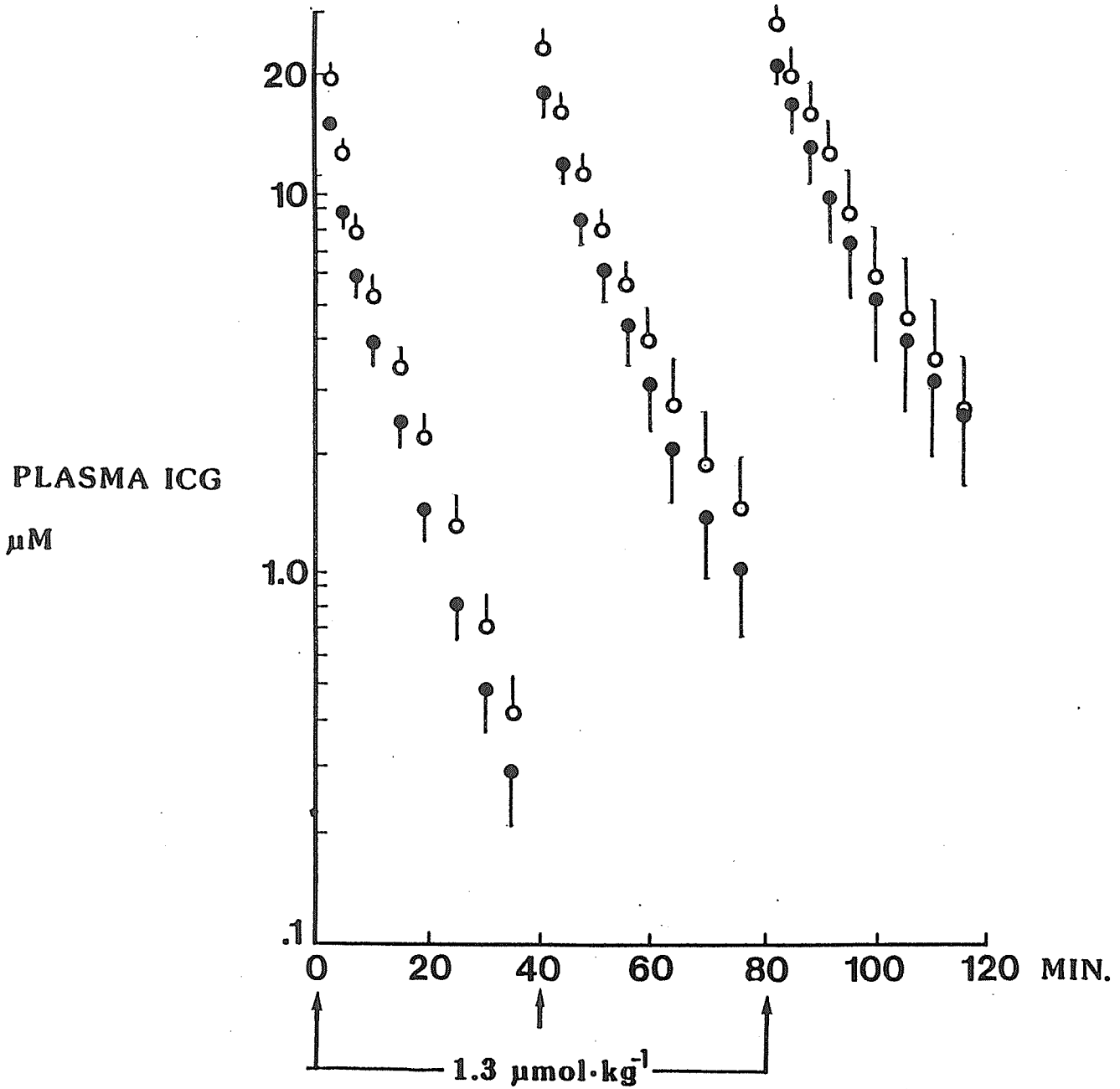
1. PLASMA ICG MEASUREMENTS AND EXTRACTION

The mean arterial and hepatic venous plasma concentrations of ICG plotted on a logarithmic scale against time for the first series (3 bolus injections) are shown in fig. 10. These curves show a definite and consistent curvature as the concentration falls. Arterial and hepatic venous curves are approximately parallel suggesting little change in extraction as the concentration decreased (see below). The apparent elimination rate constants were calculated from the slopes of the arterial plasma concentration-time curves from 3 to 12 min after each dose, assuming a one compartment open model with a first order process (50). The rate constants were

Table 3. Arterial and hepatic venous blood gases and pH during all of the experimental series. Mean ± SE.

	Initial	Intermediate	Final
Arterial			
pH	7.40 ± 0.01	7.39 ± 0.01	7.37 ± 0.02
pCO_2	26.2 ± 0.8	25.0 ± 0.9	26.2 ± 1.1
pO_2	87.4 ± 4.8	88.9 ± 6.5	94.9 ± 6.1
Hepatic venous			
pH	7.40 ± 0.01	7.37 ± 0.02	7.37 ± 0.02
pCO_2	29.1 ± 1.0	29.9 ± 1.1	30.7 ± 1.0
pO_2	34.8 ± 2.0	32.5 ± 1.7	30.8 ± 2.2

Fig. 10. Arterial (O) and hepatic venous (●) plasma concentrations of ICG plotted on a logarithmic scale against time for the first experimental series. Means ± S.E. for 5 cats.

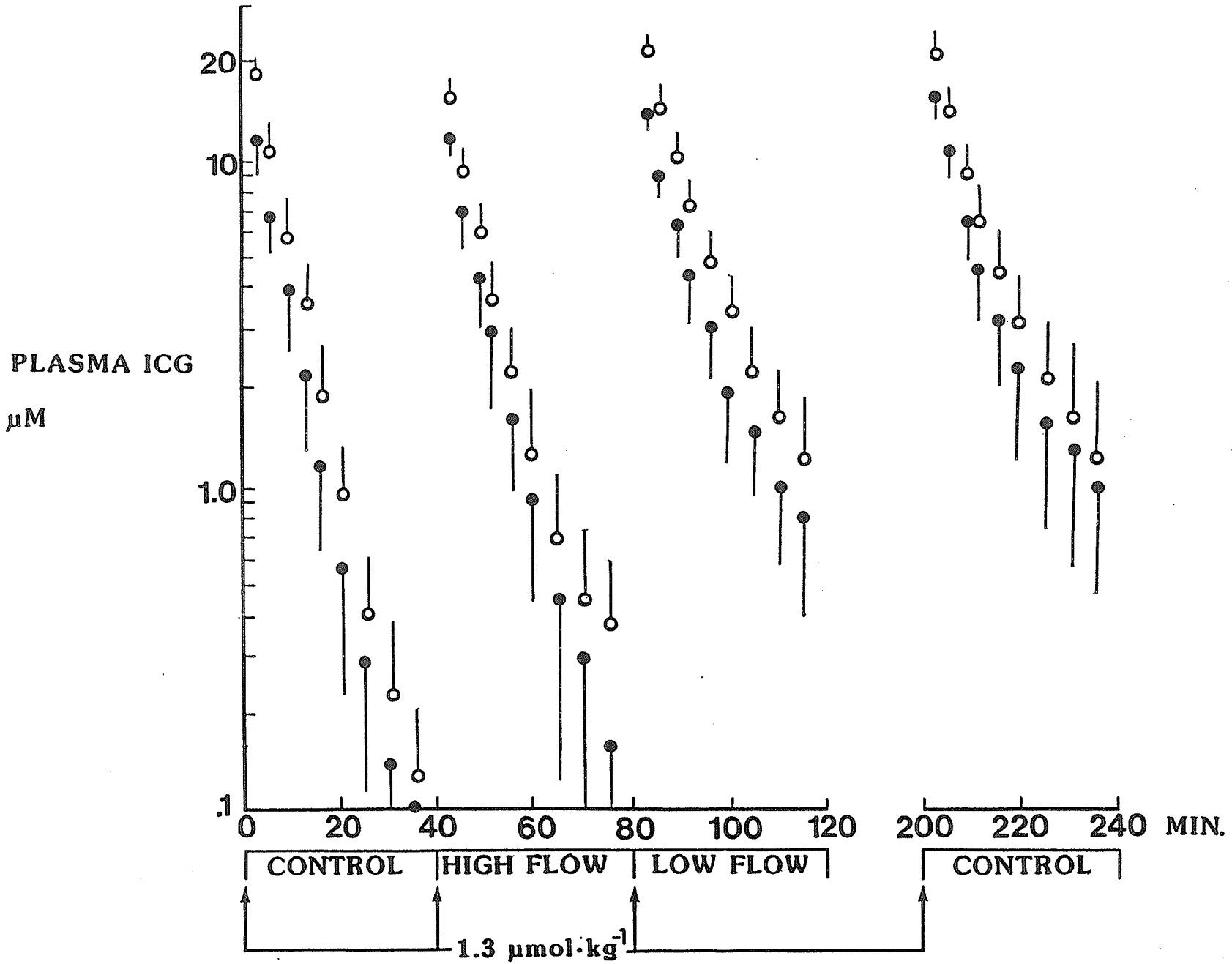

PLASMA ICG
µM
20
10
1.0
.1
0
20
40
60
80
100
120
MIN.
1.3 µmol·kg⁻¹

0.14 ±0.01, 0.11 ± 0.01 and 0.09 ± 0.01 min^{-1} for the 3 doses, respectively. All values were significantly different from each other ($p<0.01$). Plasma half-lives were 5.0 ± 0.2, 6.1 ± 0.4 and 7.7 ± 1.0 min for the three doses, respectively.

The arterial and hepatic venous plasma ICG concentrations at the different hepatic plasma flow rates in the second series of 5 cats are shown in fig. 11. The elimination rate constants were 0.22 ± 0.01, 0.18 ± 0.04, 0.13 ± 0.02 and 0.14 ± 0.02 min^{-1} for the first control, low, high and second control flow rates, respectively. These values were significantly different ($p<0.01$) with exception of the second control and low flow rate. Plasma half-lives were 2.9 ± 0.6, 4.4 ± 0.9, 6.0 ± 0.9 and 5.5 ± 1.1 min for the four doses, respectively.

The apparent volume of distribution for ICG was calculated by dividing the first dose by the extrapolated arterial plasma ICG concentration at the time of administration, subtracting reservoir volume and correcting the resultant volume for body weight and hematocrit. The apparent volume of distribution was 62 ± 6, 57 ± 6 and 48 ± 7 ml/kg for the first, second and third bolus doses, respectively. These values decreased significantly ($p<0.01$) with successive doses. The apparent volume of distribution

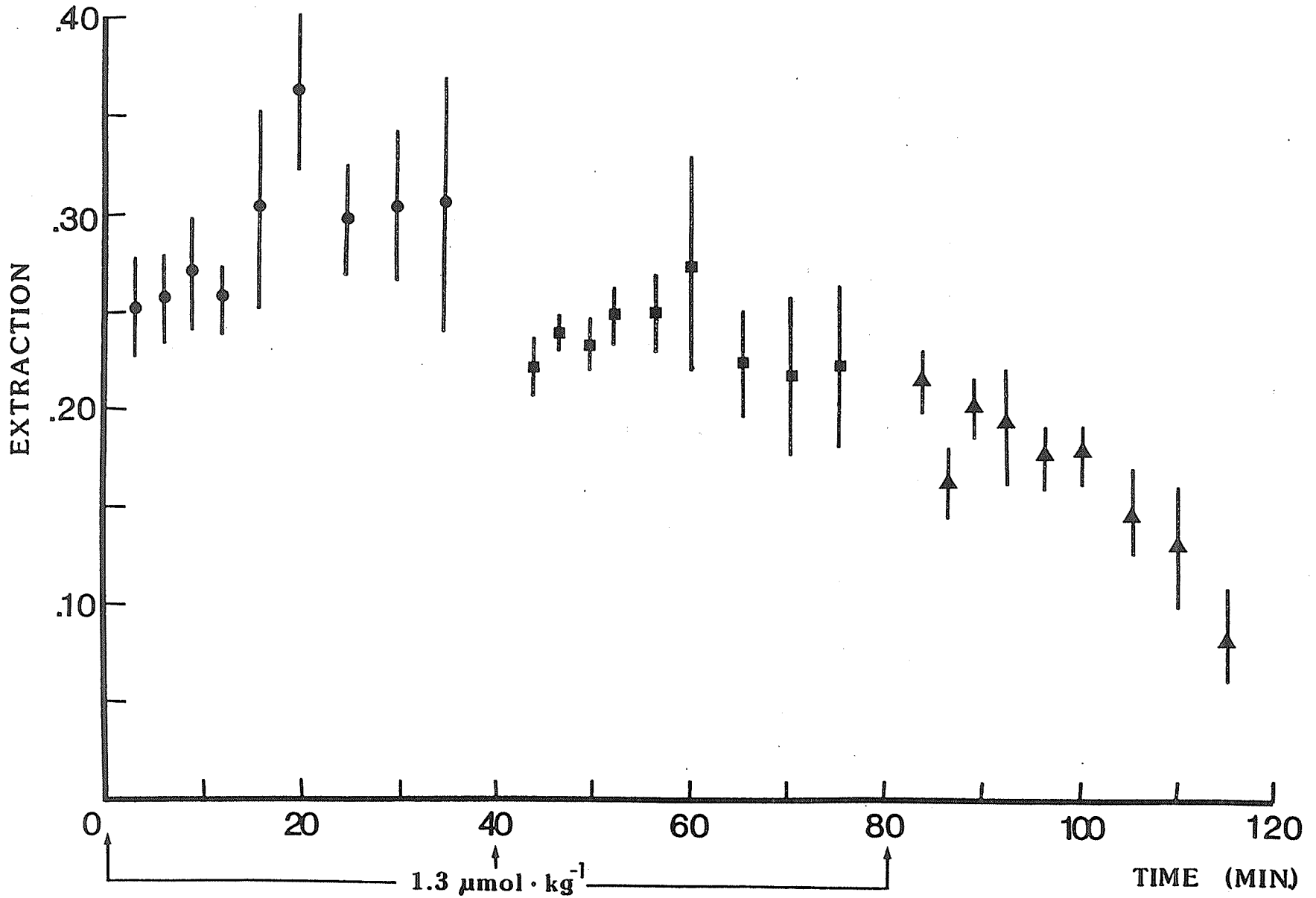
Fig. 11. Arterial (O) and hepatic venous (●) plasma concentrations of ICG plotted on a logarithmic scale against time for the second experimental series. Means ± S.E. for 5 cats.


PLASMA ICG
µM
20
10
1.0
.1
0
20
40
60
80
100
120
200
220
240
MIN.
CONTROL
HIGH FLOW
LOW FLOW
CONTROL
1.3 µmol·kg⁻¹

for ICG during the second study was 44 ± 3, 49 ± 4, 49 ± 10 and 46 ± 2 ml/kg for each of the four doses, respectively. The apparent volume of distribution in the hepatectomized animals was 36 ± 5 ml/kg. Overall, the pooled apparent volume of distribution after the first dose in the first two series was 53 ± 4 ml/kg (N=10). The apparent ICG volume of distribution was compared in 5 cats with the volume determined as above but using ^{125}I-labelled bovine serum albumin. The apparent volume of distribution of ICG was not different (48 ± 2 ml/kg, N = 5) from that determined with the labelled albumin (43 ± 3 ml/kg, N = 5). Both methods gave estimates which approximated the circulating blood volume in cats (63).

Extraction of ICG was calculated in 3 ways. Extraction for each pair of arterial (Ca) and hepatic venous (Cv) samples was calculated as (Ca-Cv)/Ca. There was a large variability in extraction as the plasma ICG concentration decreased after each dose. In some cats, extraction increased while in others it fell. Overall extraction did not change significantly as the plasma concentrations decreased following each bolus dose except for the marked decrease as the plasma concentrations fell after the third dose. The extraction of ICG after successive bolus doses during the first study is illustrated in fig. 12. For each bolus dose, the areas under the arterial (AUCa) and hepatic

Fig. 12. Indocyanine green extraction versus time plot for the three bolus doses (first series). Mean ± S.E. N = 5. ● - first dose; ■ - second dose; ▲ - third dose.


.40
.30
.20
.10
EXTRACTION
0
20
40
60
80
100
120
1.3 µmol · kg⁻¹
TIME (MIN)

venous (AUCv) concentration-time curves were calculated using the polygon formula. Mean extraction was then calculated as (AUCa - AUCv)/AUCa. For the 3 doses, mean extraction was 0.25 $\pm$ 0.02, 0.22 $\pm$ 0.02 and 0.17 $\pm$ 0.02, respectively. Extraction following the third bolus dose was significantly lower (analysis of variance, $p<0.05$) indicating extraction decreased with repetitive doses. The expected extraction was calculated as (Dose/AUCa)/measured hepatic plasma flow. For the 3 doses, mean extraction was 0.32 $\pm$ 0.02, 0.27 $\pm$ 0.02 and 0.22 $\pm$ 0.02, respectively. All values were different ($p<0.05$) and showed a significant reduction in extraction on repetition of the ICG bolus dose. In addition, these expected extractions calculated from the systemic clearances and the measured flows were significantly larger than the extractions calculated from the areas under the arterial and venous concentration-time curves. These differences may be due to variable transit times or extrahepatic removal of ICG (see Discussion). A negative correlation is shown between extraction and the amount of ICG remaining to be excreted by the liver ($r= -0.96$) in fig. 13.

The results of the extraction ratio calculated as (Ca-Cv)/Ca during the second study are shown in fig. 14. The high blood flow rate decreased extraction while it was increased during the low flow rate. Extraction during the

Fig. 13. Indocyanine green extraction versus amount of indocyanine green in the liver for the three bolus doses (first series). Means ± SE, N = 5

● - (Dose/AUC_a)/Measured hepatic plasma flow

■ - (AUC_a/AUC_{hv})/AUC_a

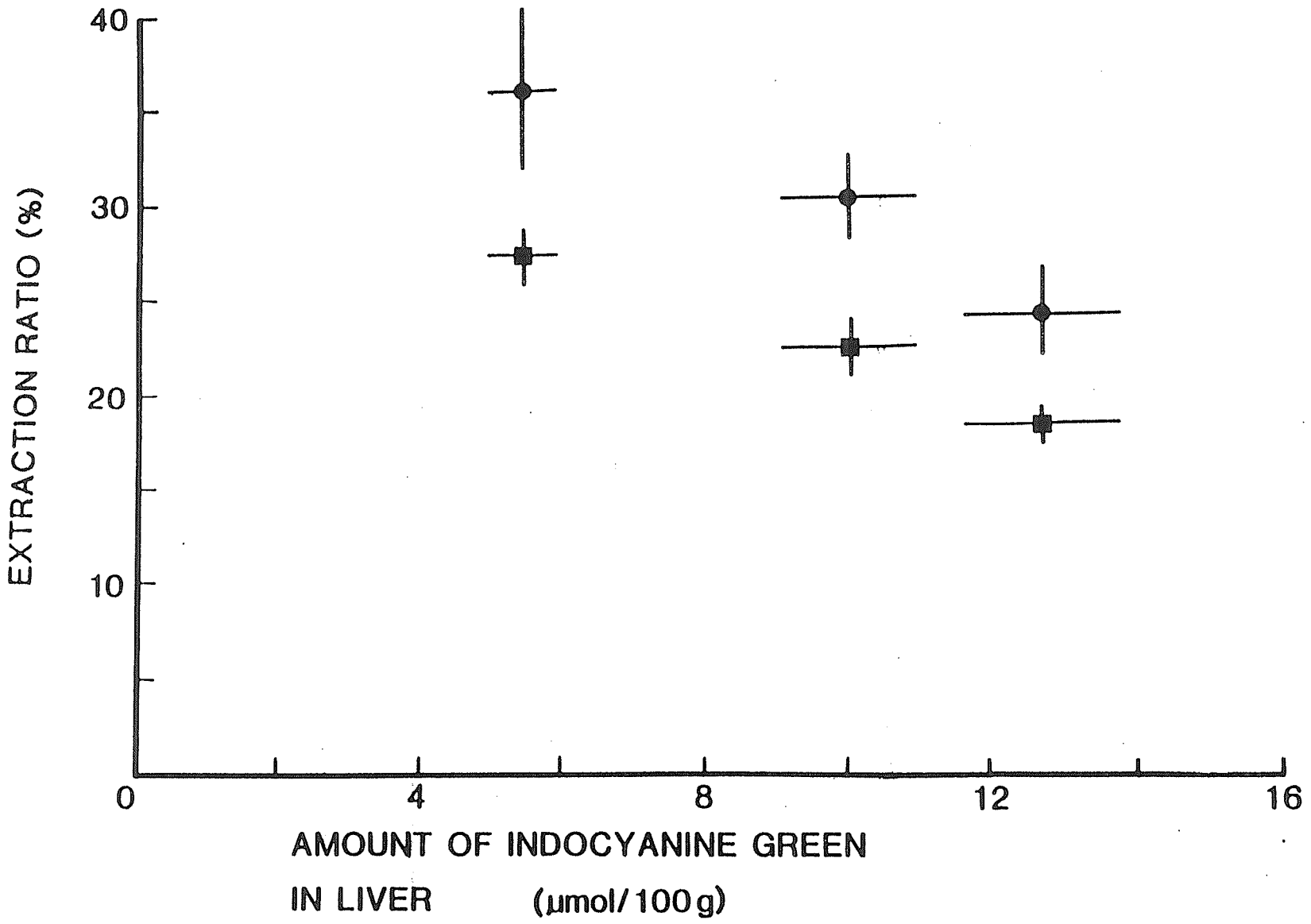
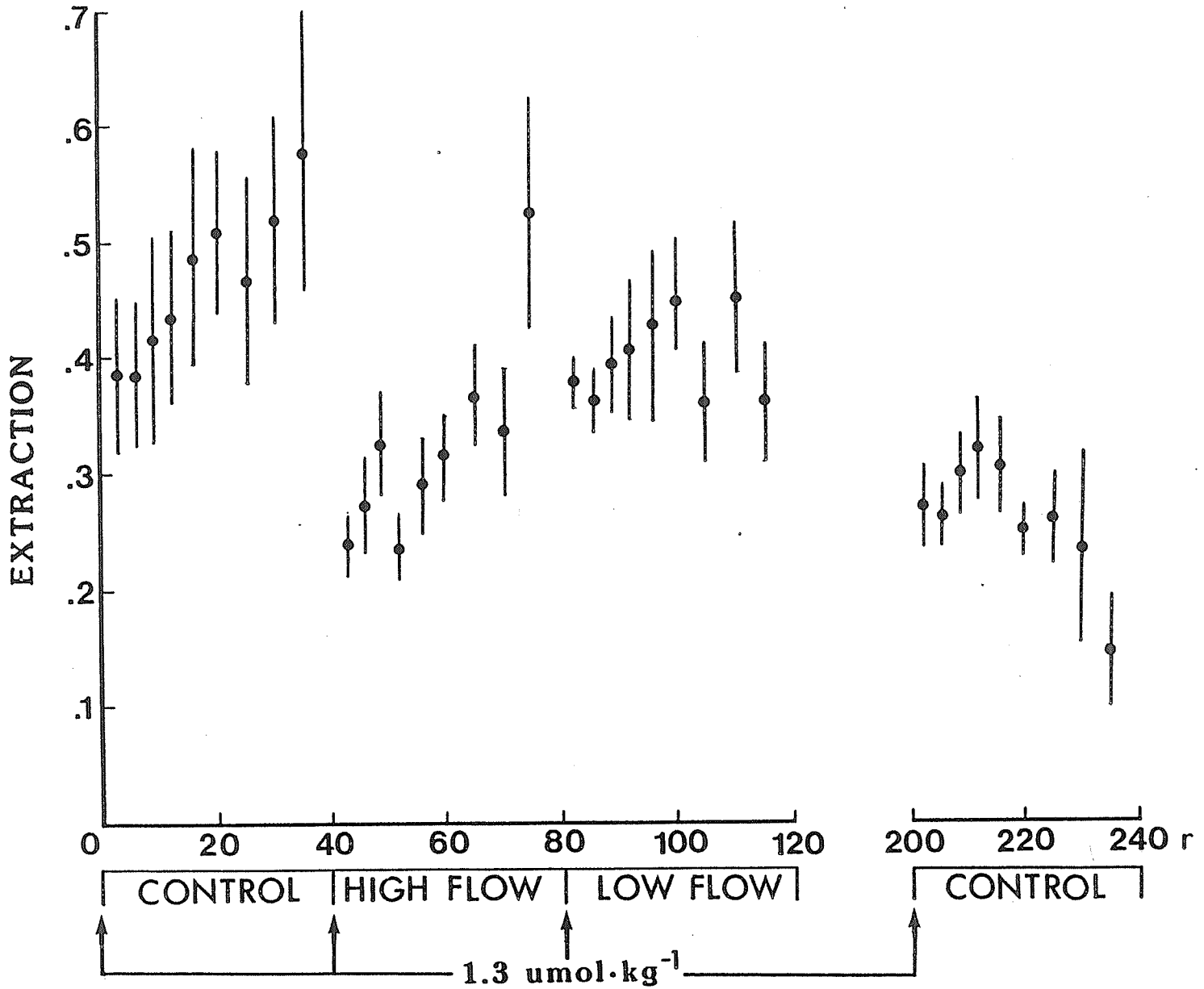

40
30
20
10
EXTRACTION RATIO (%)
0
4
8
12
16
AMOUNT OF INDOCYANINE GREEN
IN LIVER (μmol/100g)

Fig. 14. Indocyanine green extraction versus time plot during the second series of experiments (altered flow). Mean $\pm$ S.E. N = 5.


EXTRACTION
.7
.6
.5
.4
.3
.2
.1
0
20
40
60
80
100
120
200
220
240 r
CONTROL
HIGH FLOW
LOW FLOW
CONTROL
1.3 umol·kg⁻¹

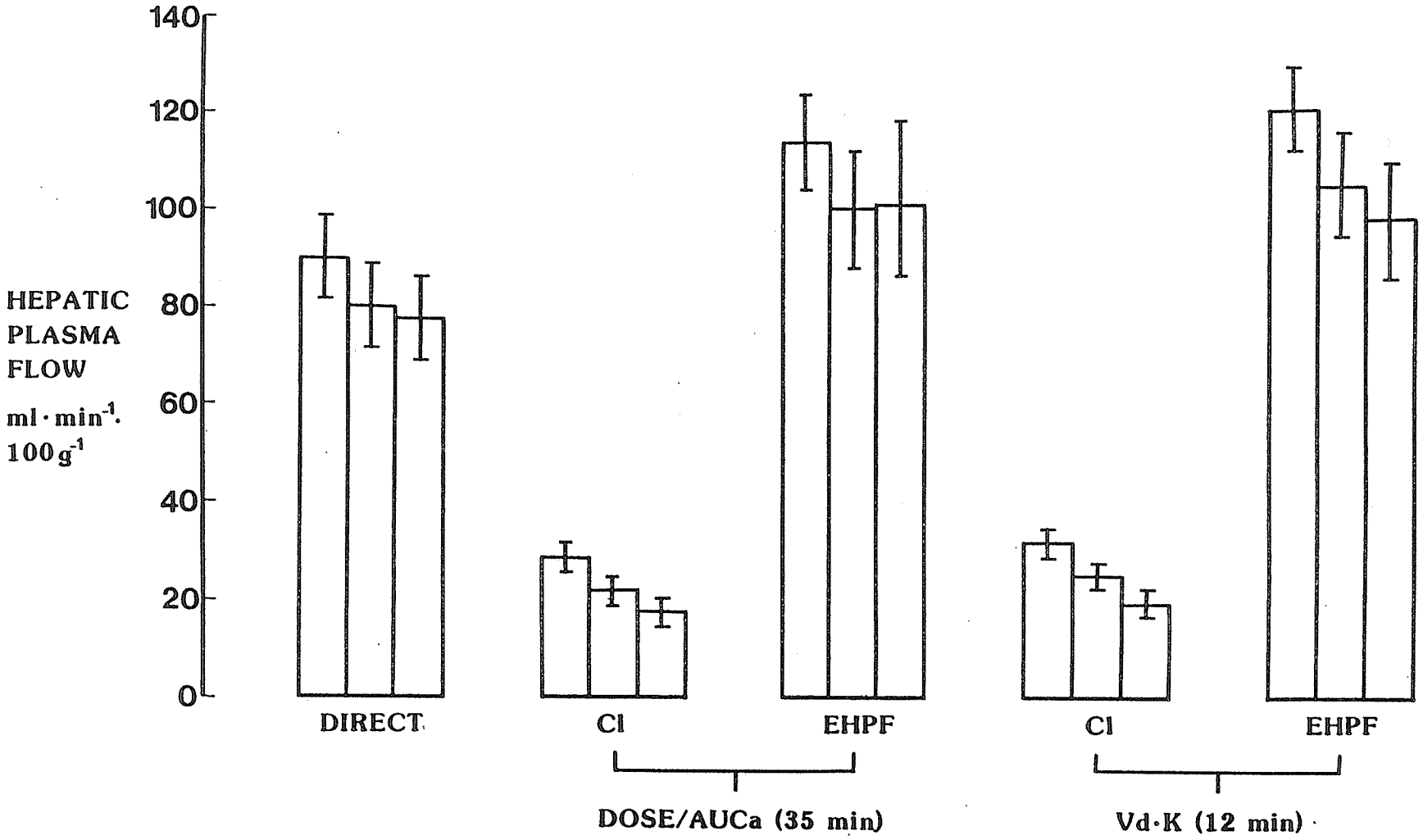
last bolus dose (control flow) was lower than the first bolus dose at control flow and extraction at the high blood flow rate was significantly lower than that of the first control and low blood flow rates. Similarly, extraction at the low blood flow rate was greater than that of the high and last control blood flow rates ($p<0.05$). Mean extraction calculated by the area method was 0.34 ± 0.06 for the first bolus dose at control flow, 0.23 ± 0.03 for the second bolus at high flow, 0.34 ± 0.03 for the third bolus at low flow and 0.25 ± 0.03 for the fourth bolus dose at control flow. Analysis of variance indicated that the extraction of ICG at the high flow and final control flow rate was lower ($p<0.05$) than the first control and low blood flow periods. Finally, expected extractions, calculated as (dose/AUCa)/measured hepatic plasma flow for the four doses were 0.42 ± 0.05, 0.31 ± 0.03, 0.49 ± 0.03 and 0.30 ± 0.04, respectively. These extractions were significantly different with the exception of the high and final control blood flow rates. The extractions calculated from the systemic clearances and the measured flows were significantly larger than the extractions calculated by the area method.

2. SYSTEMIC ICG CLEARANCE

Clearance of ICG was calculated in two ways. In one

method it was calculated as Dose/AUCa where AUCa was measured for the whole concentration curve after ICG (35 min). Clearance during the first, second and third doses was 29 ± 3, 22 ± 3 and 17 ± 2 ml/min/100g liver, respectively. In the second method it was calculated as Vd • k; where Vd is the apparent volume of distribution and k is the apparent elimination rate constant calculated over the period 3-12 min after each dose. Clearance during the first, second and third dose was 32 ± 3, 24 ± 3 and 19 ± 3 ml/min/100g liver for the 3 doses in the first series, respectively. The two calculations gave similar results. ICG clearance decreased with successive doses. Furthermore, these values represent the estimates of hepatic plasma flow which would be obtained if extraction of ICG was assumed to be 100%. In each case, a better estimate of hepatic plasma flow was then calculated by dividing clearance by mean extraction calculated over the same time periods. The directly measured hepatic plasma flows and the results of these calculations for the first series are presented in fig. 15. All estimates of clearance and hepatic plasma flow were significantly different from the directly measured flows ($P<0.01$; by blocked ANOVA). The S.E.s in fig. 15 mainly reflect differences between cats. Systemic clearances were similar by the two calculations but grossly underestimated hepatic plasma flow. When the clearances were corrected for the

Fig. 15. Hepatic plasma flows measured directly and calculated for each of the 3 doses of ICG (groups of 3 bars) in the first series. ICG clearance (Cl) was calculated in two ways and these values represent the estimates of hepatic plasma flows if extraction is assumed to be 100%. Another estimate of hepatic plasma flows (EHPF) was calculated as clearance divided by mean extraction over the appropriate time period. Means $\pm$ S.E. for 5 cats. All calculated values are significantly different from the measured values for the corresponding dose ($P<0.01$; blocked analysis of variance).


140
120
100
80
60
40
20
0
HEPATIC PLASMA FLOW
ml · min-1 · 100 g-1
DIRECT
Cl
EHPF
Cl
EHPF
DOSE/AUCa (35 min)
Vd·K (12 min)

appropriate extraction, the resulting estimates of hepatic plasma flow consistently and significantly exceeded the directly measured flows. The individual calculated flows plotted against the measured flows for each dose in each cat are shown in fig. 16. It can be seen that estimated hepatic flow approached the true hepatic flow on only a few occasions.

For the second study, clearance of ICG was again calculated using two methods, Vd • k and DOSE/AUCa. The first method yielded clearance values of 39 ± 6, 40 ± 7, 25 ± 2 and 27 ± 3 ml/min/100g liver for the 4 doses, respectively, while the second method gave values of 37 ± 6, 39 ± 6, 22 ± 2 and 25 ± 3 ml/min/100g liver, respectively. The two methods resulted in similar values. ICG clearance did not increase significantly when hepatic blood flow was increased. During the low blood flow period clearance decreased significantly from that of the first control and high blood flow periods. As flow was returned to the final control rate, clearance of ICG remained less than the first control and high blood flow rates. The clearance values greatly underestimated hepatic plasma flow (fig. 17). When the clearance values were corrected by the mean extraction calculated over the same time periods, a better estimate of hepatic plasma flow resulted, but the estimated plasma flows generally exceeded the

Fig. 16. Estimated hepatic plasma flows (EHPF) from ICG administration plotted against directly measured flows (Meas. HPF) for each of 3 doses in each of 5 animals (first series). The line of identity bisects the two axes.

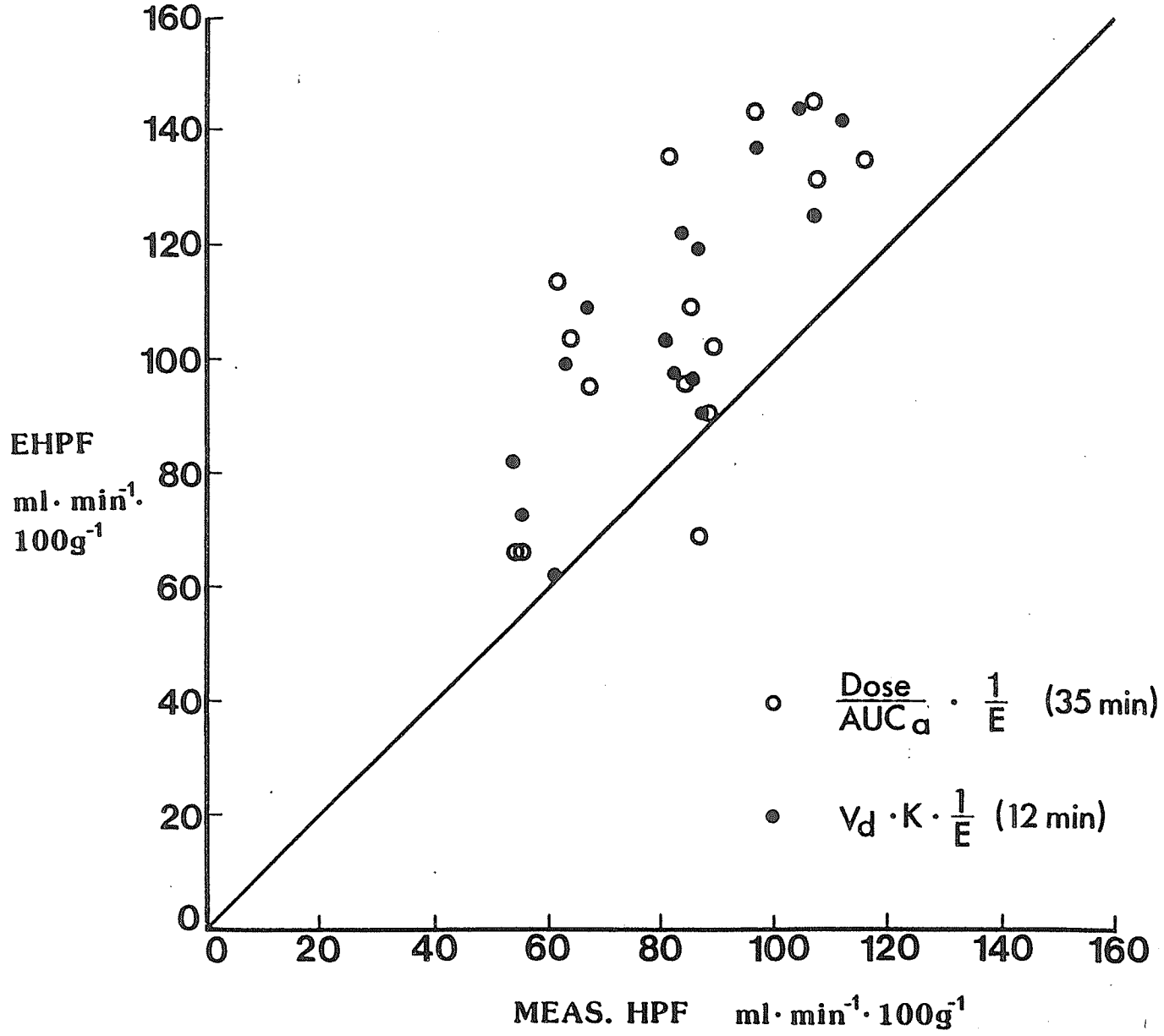
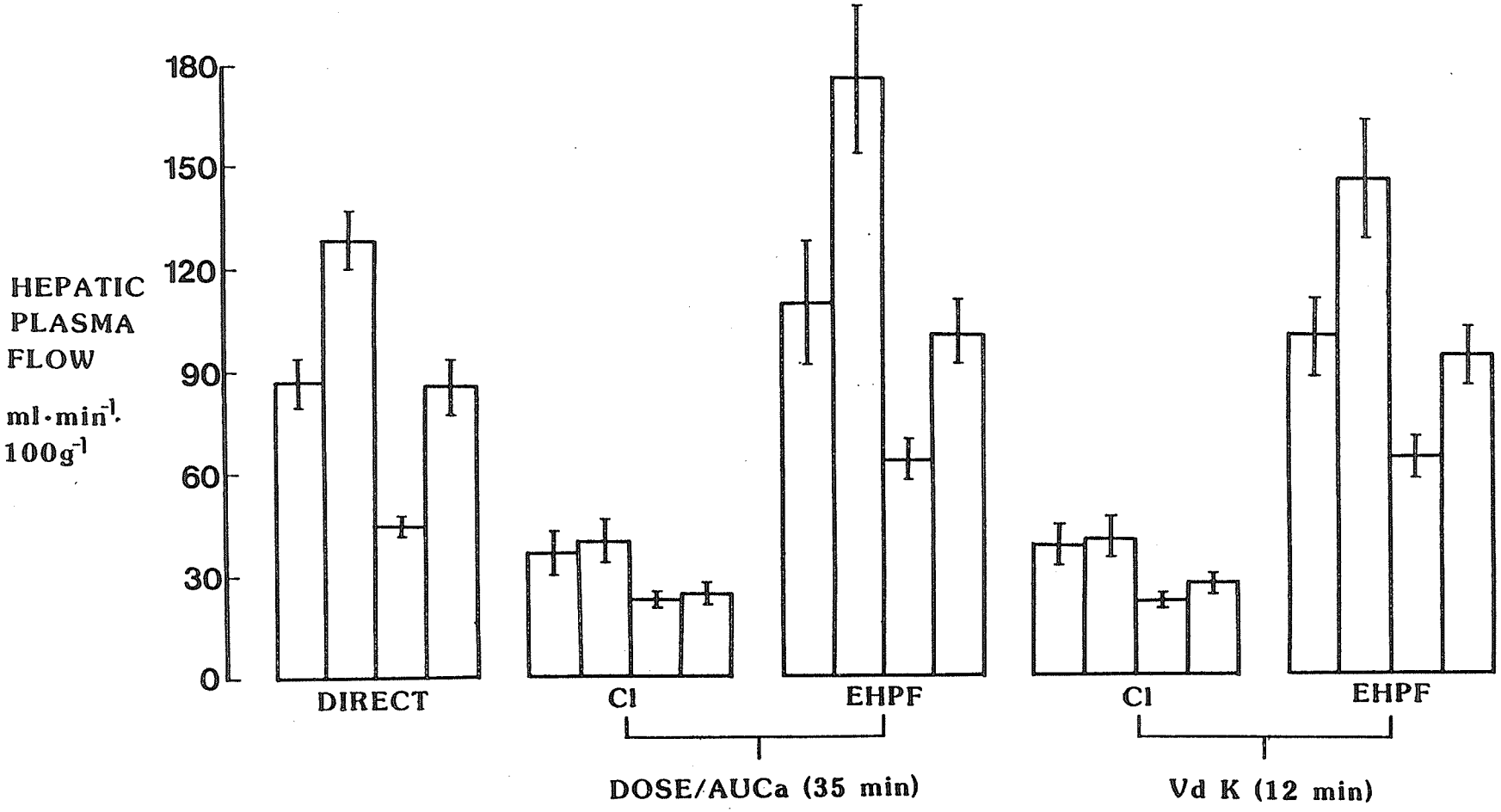

160
140
120
100
80
60
40
20
0
EHPF
ml · min-1 · 100g-1
0
20
40
60
80
100
120
140
160
MEAS. HPF ml · min-1 · 100g-1
Dose / AUC a · 1/E (35 min)
Vd · K · 1/E (12 min)

Fig. 17. Hepatic plasma flows measured directly and calculated for each of the 4 doses of ICG (second series). The data were calculated as in Fig. 15. Means $\pm$ S.E. for 5 cats.


HEPATIC
PLASMA
FLOW
ml·min-1·
100g-1
180
150
120
90
60
30
0
DIRECT
Cl
EHPF
Cl
EHPF
DOSE/AUCa (35 min)
Vd K (12 min)

measured flows. The estimates were also highly variable (fig. 18). There was no difference between the two methods for estimating hepatic plasma flow. When the first bolus dose from the first and second series were pooled, estimated plasma flow was much greater than the measured flow, 111 $\pm$ 7 and 88 $\pm$ 5 ml/min/100g liver, respectively ($p<0.01$).

3. HEPATIC ICG CLEARANCE

Hepatic ICG uptake was calculated as flow times the arteriovenous concentration difference. This rate is plotted against arterial ICG concentration in fig. 19 (first series) and fig. 20 (second series). The rate of uptake was linearly related to the arterial plasma concentrations during the first series of experiments ($r^2>0.98$; $P<0.001$). The uptake rate per unit arterial concentration decreased with successive doses. The slopes are the hepatic clearances of ICG. These hepatic clearances were significantly different ($p<0.05$) from the systemic clearances calculated above during the first two doses of the first series. Cumulative uptake by the liver was calculated from the area under the uptake rate plotted against time curve. Total area under the hepatic uptake curves represented 82%, 80% and 82% of each dose. However, as noted earlier, hepatic uptake calculated as the sum of

Fig. 18. Estimated hepatic plasma flows (EHPF) calculated from ICG data plotted against directly measured flows (Meas. HPF) for all of the altered flow periods including the control periods. The line of identity bisects the two axes.

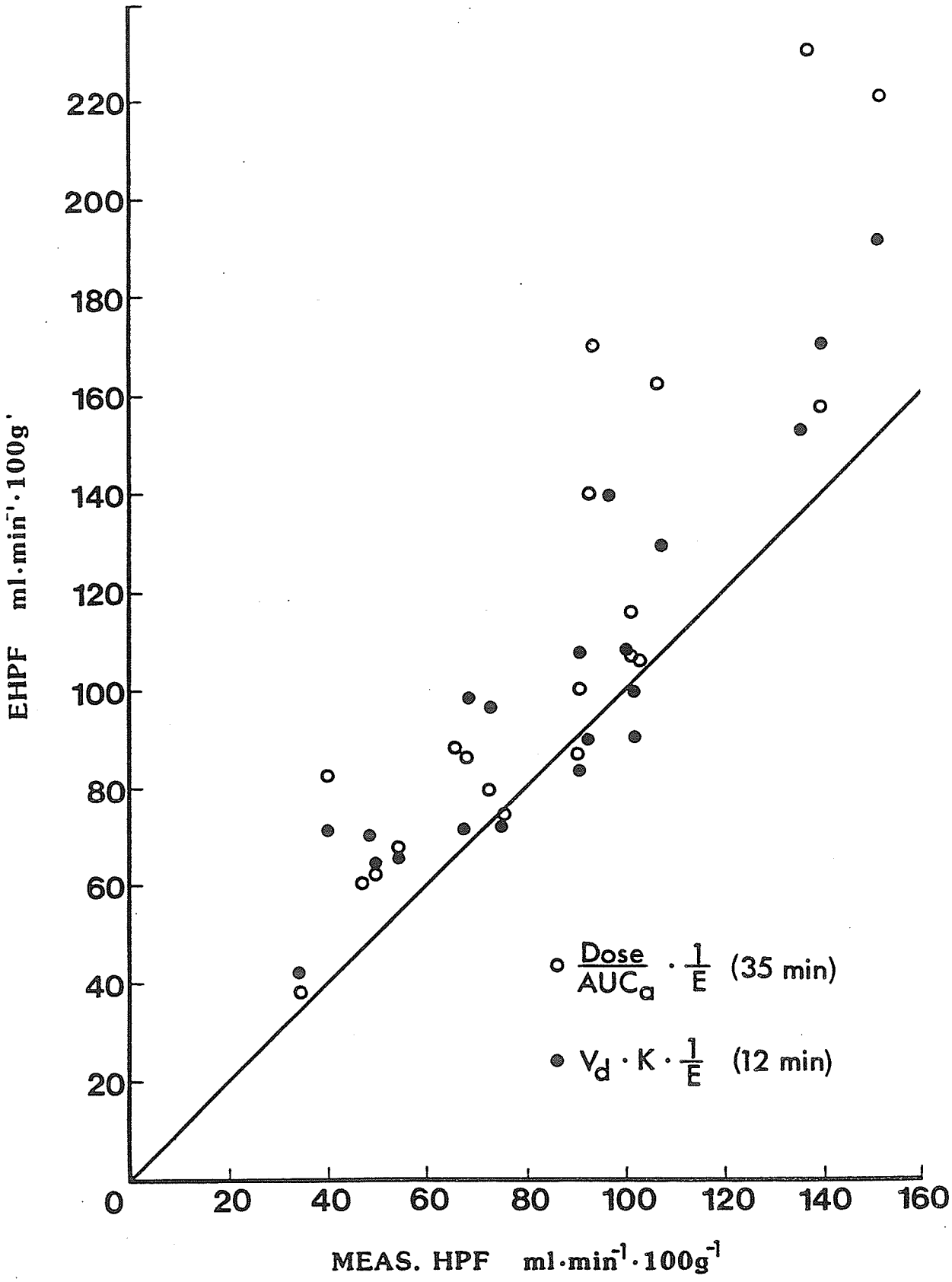

EHPF ml·min⁻¹·100g⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
MEAS. HPF ml·min⁻¹·100g⁻¹
Dose / AUCa · 1/E (35 min)
Vd · K · 1/E (12 min)

Fig. 19. The mean hepatic uptake rate for each of the three doses of ICG plotted against mean arterial plasma concentration for the first series of experiments. The slopes, given as numbers on the figures, are the hepatic clearances of ICG for the first, second and third doses, respectively.

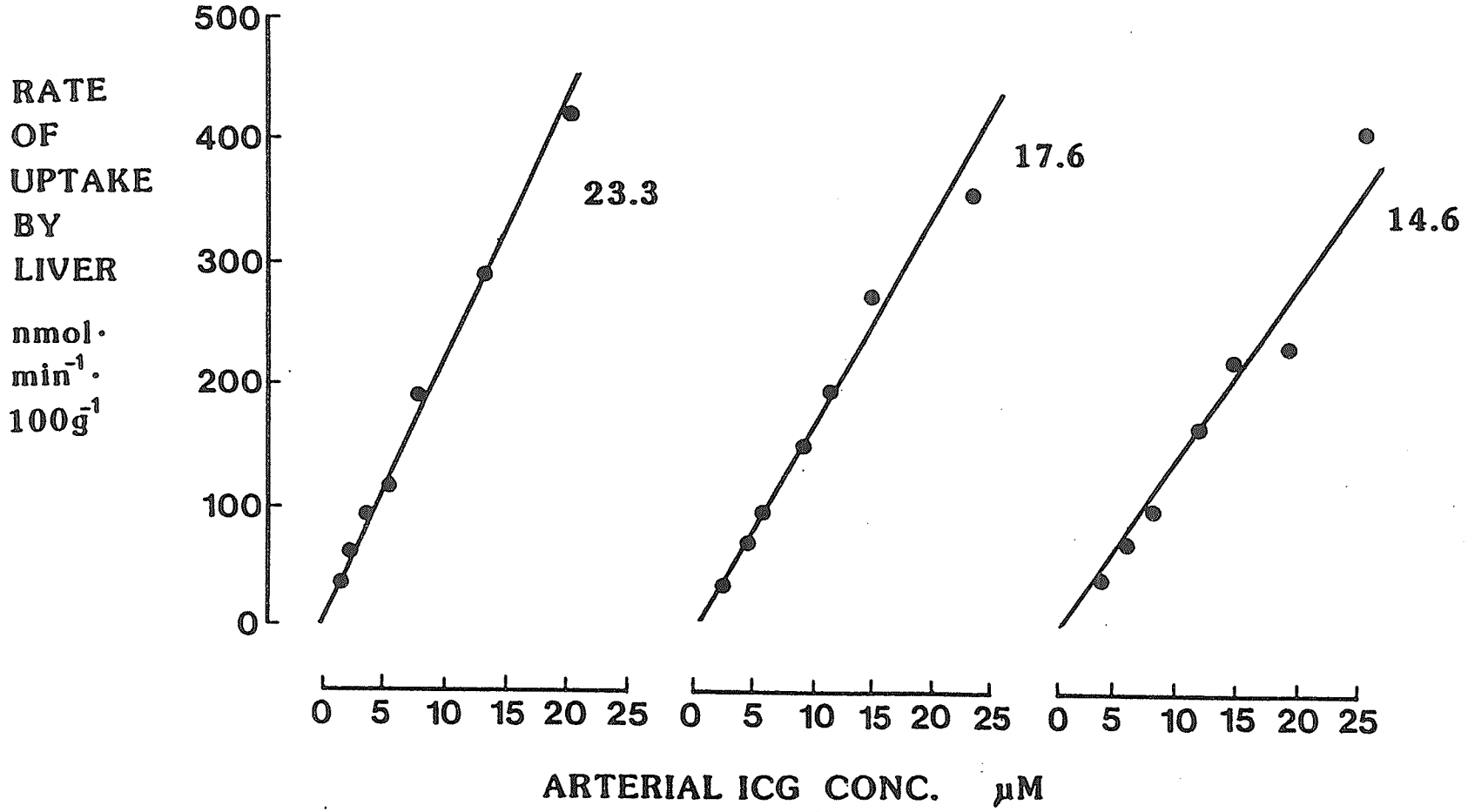
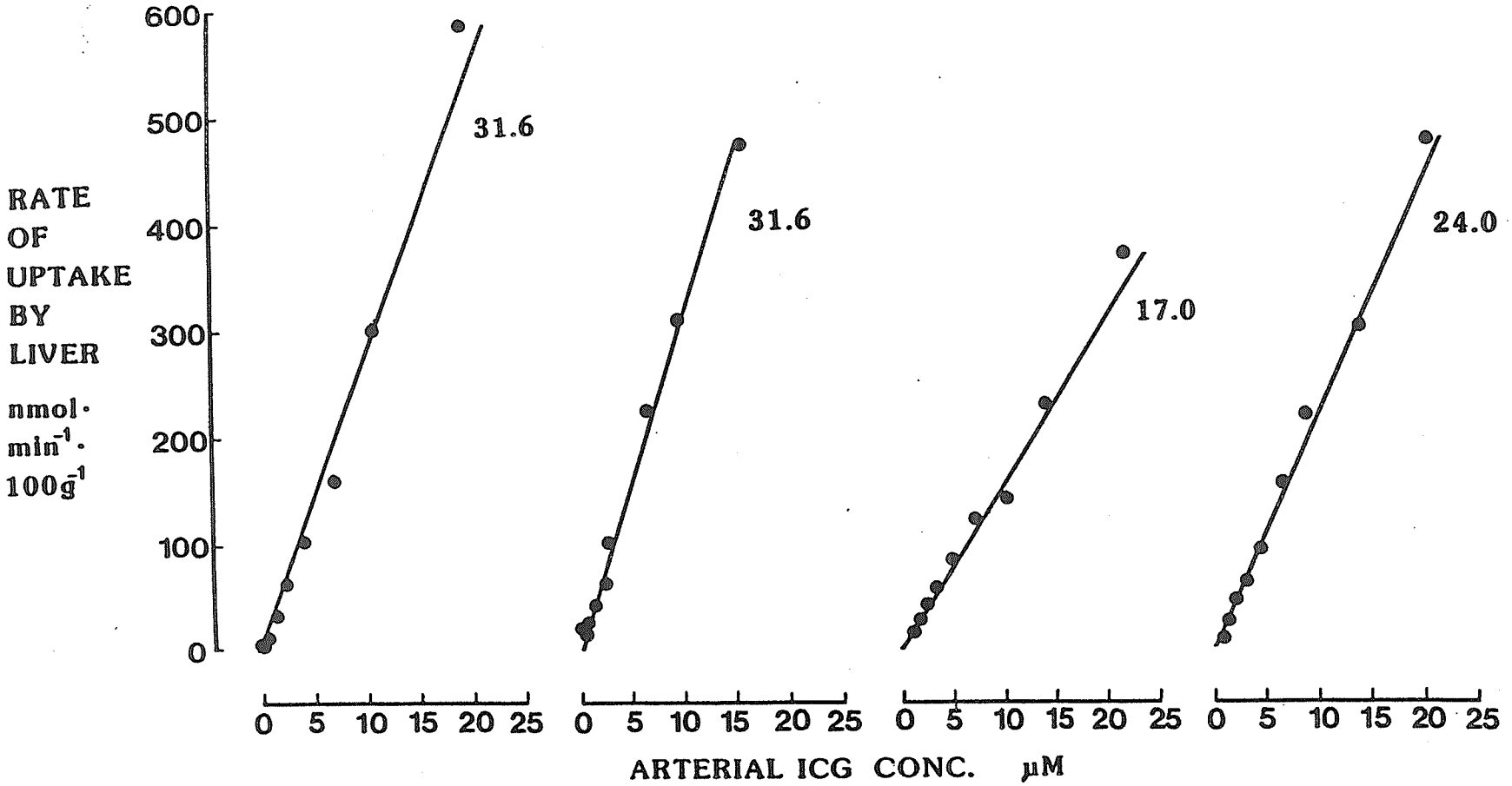

RATE OF UPTAKE BY LIVER
nmol · min⁻¹ · 100g⁻¹
500
400
300
200
100
0
23.3
17.6
14.6
0 5 10 15 20 25
0 5 10 15 20 25
0 5 10 15 20 25
ARTERIAL ICG CONC. µM

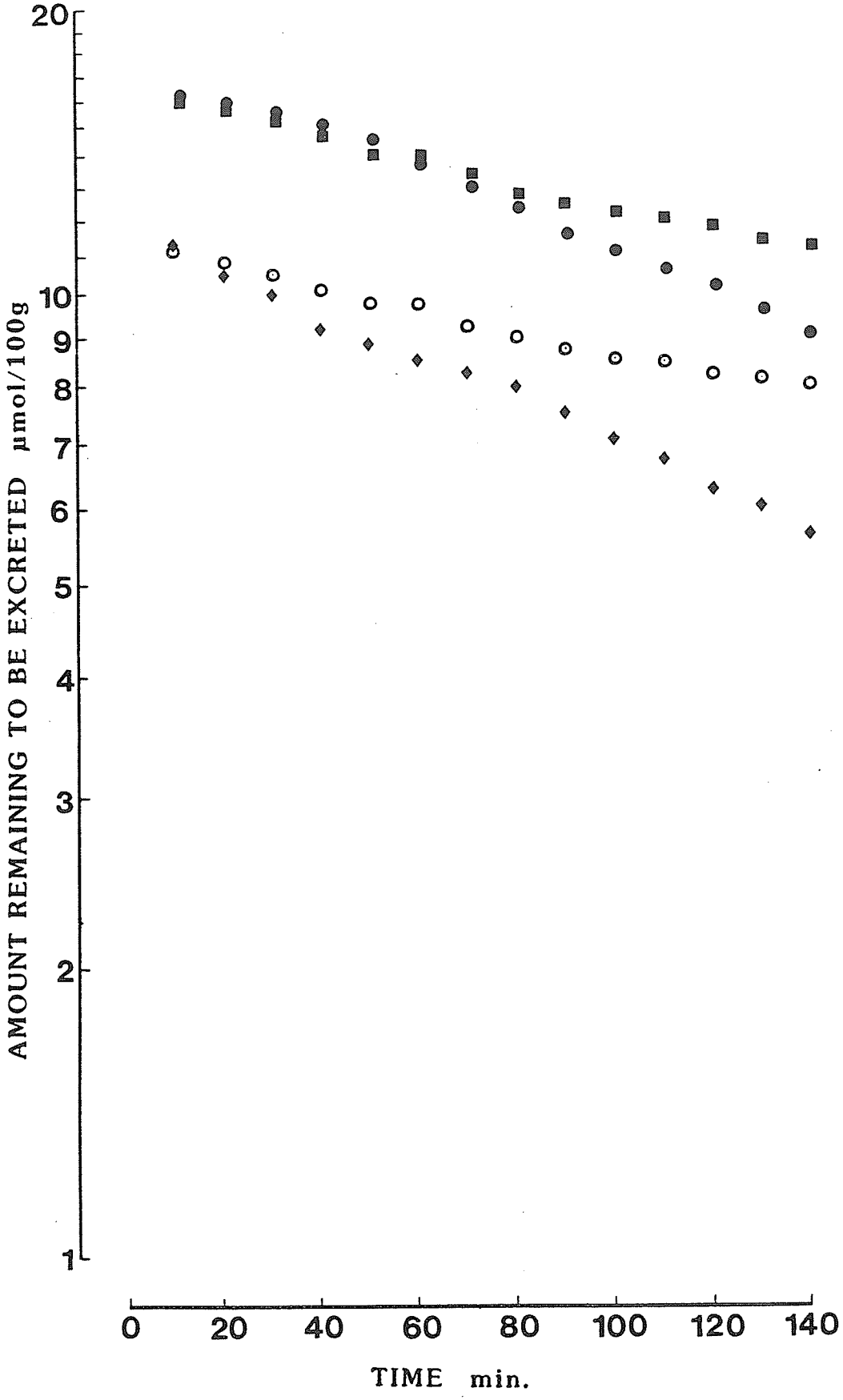
Fig. 20. The mean hepatic uptake rate for each dose of ICG is plotted against mean arterial plasma concentration for the second series of experiments (altered flow). The slopes, given as numbers on the figures, are the hepatic clearances of ICG starting with the first control, high, low and final control flow rates, respectively.


RATE OF UPTAKE BY LIVER
nmol · min⁻¹ · 100g⁻¹
600
500
400
300
200
100
0
31.6
31.6
17.0
24.0
0 5 10 15 20 25
0 5 10 15 20 25
0 5 10 15 20 25
0 5 10 15 20 25
ARTERIAL ICG CONC. µM

ICG in liver and cumulated bile at 6 h (first series) was 97% of the administered dose. The hepatic uptake rate was also linearly related to the arterial plasma concentration at the different flows ($r>0.94$; $p<0.001$). The hepatic clearances in fig. 20 were not significantly different from the calculated systemic clearances during both control blood flow periods of the second series. However, they were much lower ($p<0.05$) during the high and low blood flow periods. Cumulative uptake by the liver was also calculated from the area under the hepatic uptake rate plotted against time curve. Total area represented 96%, 99%, 82% and 101% at the control, high, low and final control flow rates, respectively for each dose. However, hepatic uptake calculated as the sum of ICG in liver and cumulated bile at 4 h was 87% of the administered dose.

In 4 cats from the first study, bile was collected for more than 2 h after the final dose of ICG had been taken up by the liver. The biliary elimination rate constant for ICG was calculated from the slope of a plot of amount remaining to be excreted against time (50). The amount remaining to be excreted was calculated as the dose administered (per 100g liver) minus the amount excreted into the bile (per 100g liver). The biliary excretion rate constant (fig. 21) was 0.004 ± 0.001 min^{-1} ($N = 4$).

Fig. 21. Amount of ICG remaining to be excreted into bile plotted on a logarithmic scale against time for the first experimental series. Individual values for 4 cats are shown by the 4 symbols.


AMOUNT REMAINING TO BE EXCRETED µmol/100g
20
10
9
8
7
6
5
4
3
2
1
0
20
40
60
80
100
120
140
TIME min.

4. HEPATECTOMIZED CATS

Extrahepatic elimination was investigated in hepatectomized cats (N = 5). Mean arterial pressure was 86 ± 6 mm Hg and mean hematocrit was 32 ± 1%. The arterial plasma concentrations of ICG plotted on a logarithmic scale against time after a single 1.3 umol/kg body weight bolus injection of ICG is shown in fig. 22. The extrahepatic elimination rate constant, calculated from the slopes of the arterial plasma concentration-time curve from 3 to 12 min after the administration of ICG, was 0.018 ± 0.001 min^{-1} (N = 5). The corresponding plasma half-life of ICG was 38.5 min. The kidney ICG content accounted for 13 ± 2% of the administered dose (N = 5). In a total of 10 cats (5 from series 3 and 5 randomly selected from series 1 and 2) only a trace amount of ICG was found in the urine of 1 cat.

Estimated hepatic plasma flow was recalculated using the Vd • k formula and subtracting the extrahepatic apparent elimination rate constant of 0.018. The results are plotted in fig. 23 for the first series and fig. 24 for the second series. There was no difference between the estimated and measured hepatic plasma flows in either series. The individual values, shown in figs. 25 and 26 for the first and second series of experiments, were found to be highly variable.

Fig. 22. Arterial plasma concentrations of ICG plotted on a logarithmic scale against time for hepatectomized cats. Mean $\pm$ SE, N = 5.

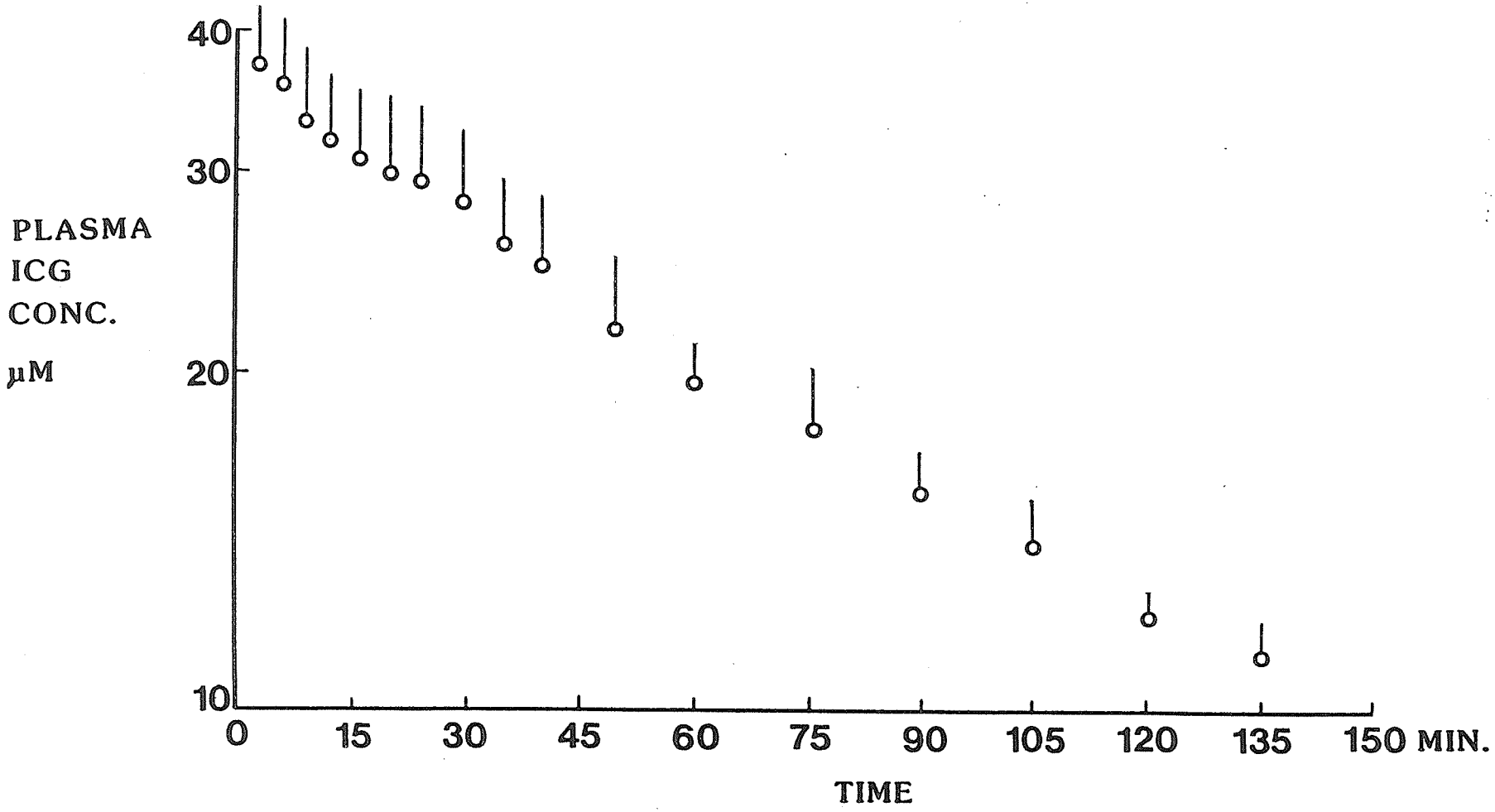

PLASMA
ICG
CONC.
µM
40
30
20
10
0
15
30
45
60
75
90
105
120
135
150 MIN.
TIME

Fig. 23. Hepatic plasma flow measured directly and calculated for each of the three doses of ICG using k (the apparent elimination rate constant) corrected for the extrahepatic uptake rate for the first series of experiments. Mean $\pm$ S.E. N = 5.

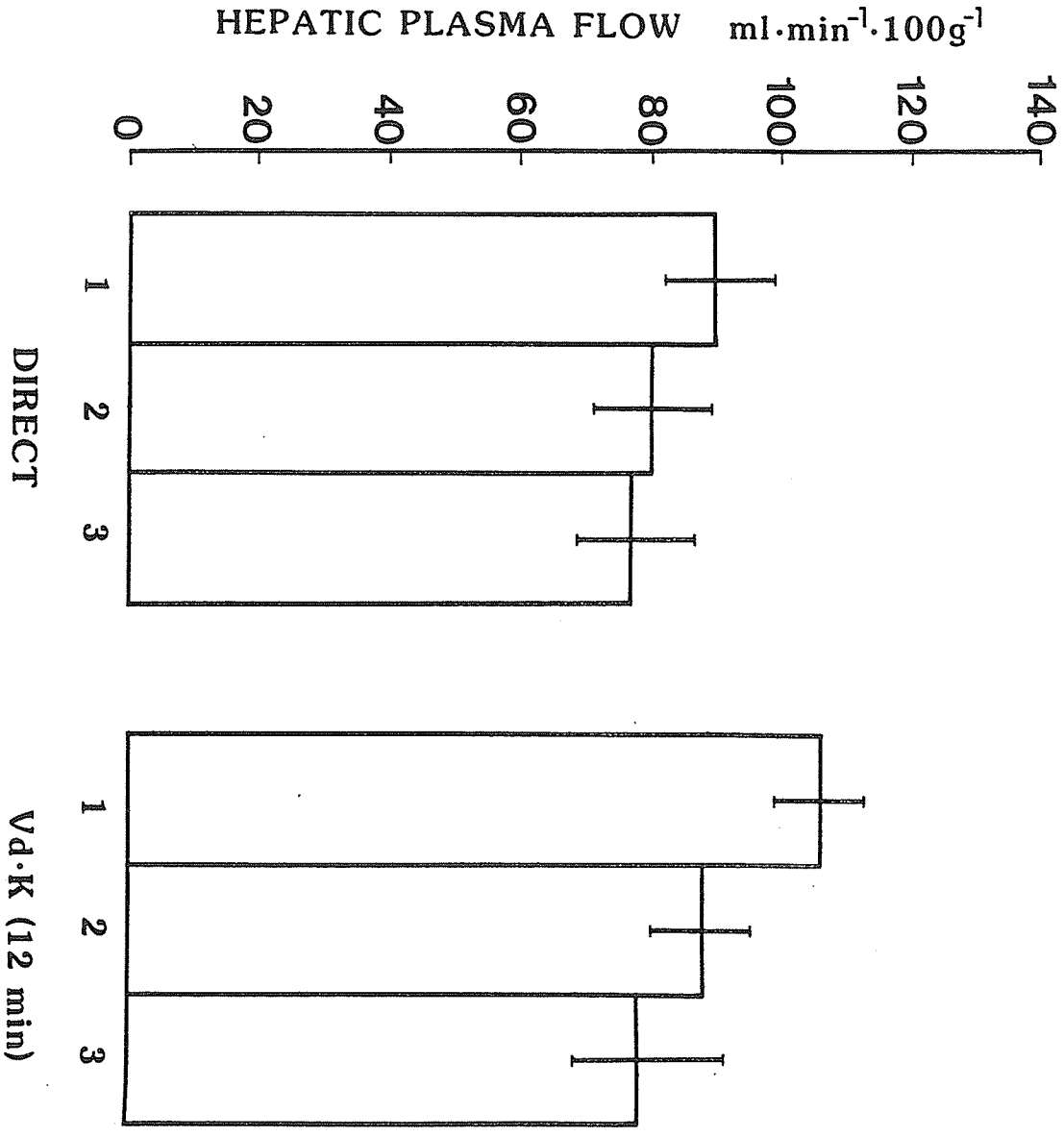

HEPATIC PLASMA FLOW ml·min⁻¹·100g⁻¹
0
20
40
60
80
100
120
140
DIRECT
1
2
3
Vd·K (12 min)
1
2
3

Fig. 24. Hepatic plasma flows measured directly and calculated for each of the four doses of ICG using k (the apparent elimination rate constant) corrected for the extrahepatic uptake rate for the second series of experiments. Mean $\pm$ S.E. N = 5. C - Control flow; H - High flow; L - Low flow.

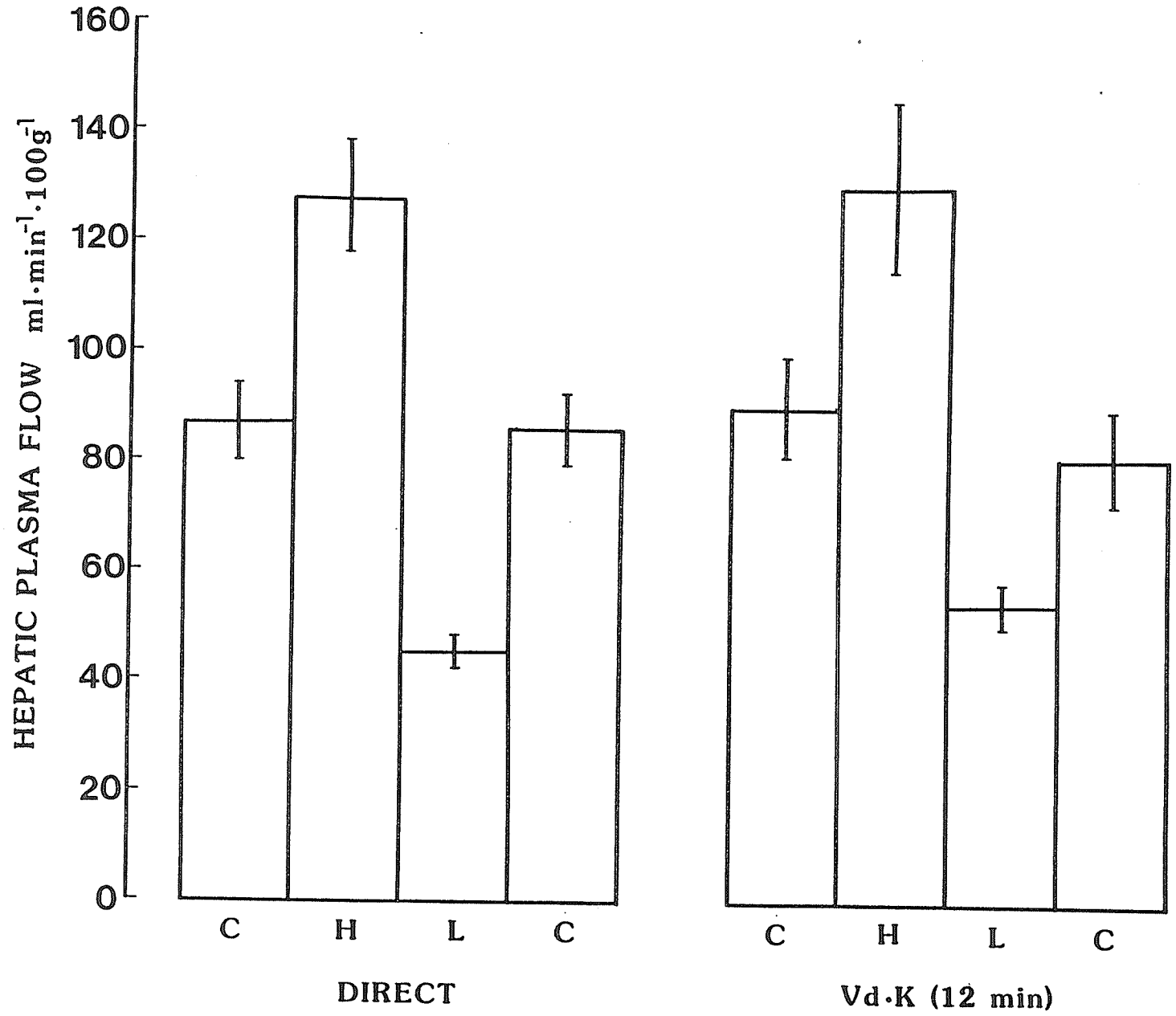

HEPATIC PLASMA FLOW ml·min⁻¹·100g⁻¹
160
140
120
100
80
60
40
20
0
C
H
L
C
DIRECT
C
H
L
C
Vd·K (12 min)

Fig. 25. Estimated hepatic plasma flows (EHPF) calculated using k (the apparent elimination rate constant) corrected for the extrahepatic uptake rate plotted against directly measured hepatic plasma flows (Meas. HPF) for each of the three doses (first series of experiments). The line of identity bisects the two axes.

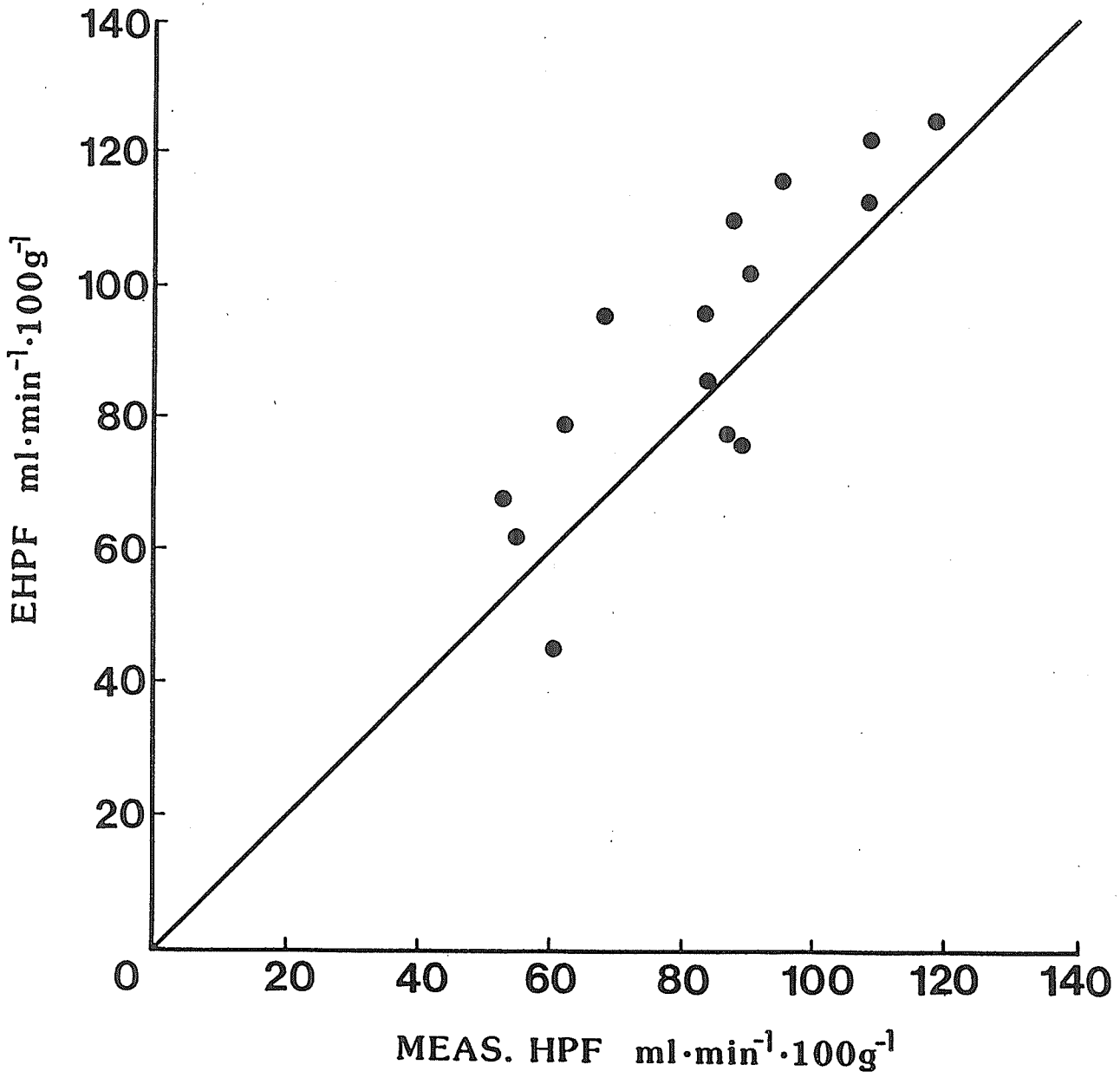
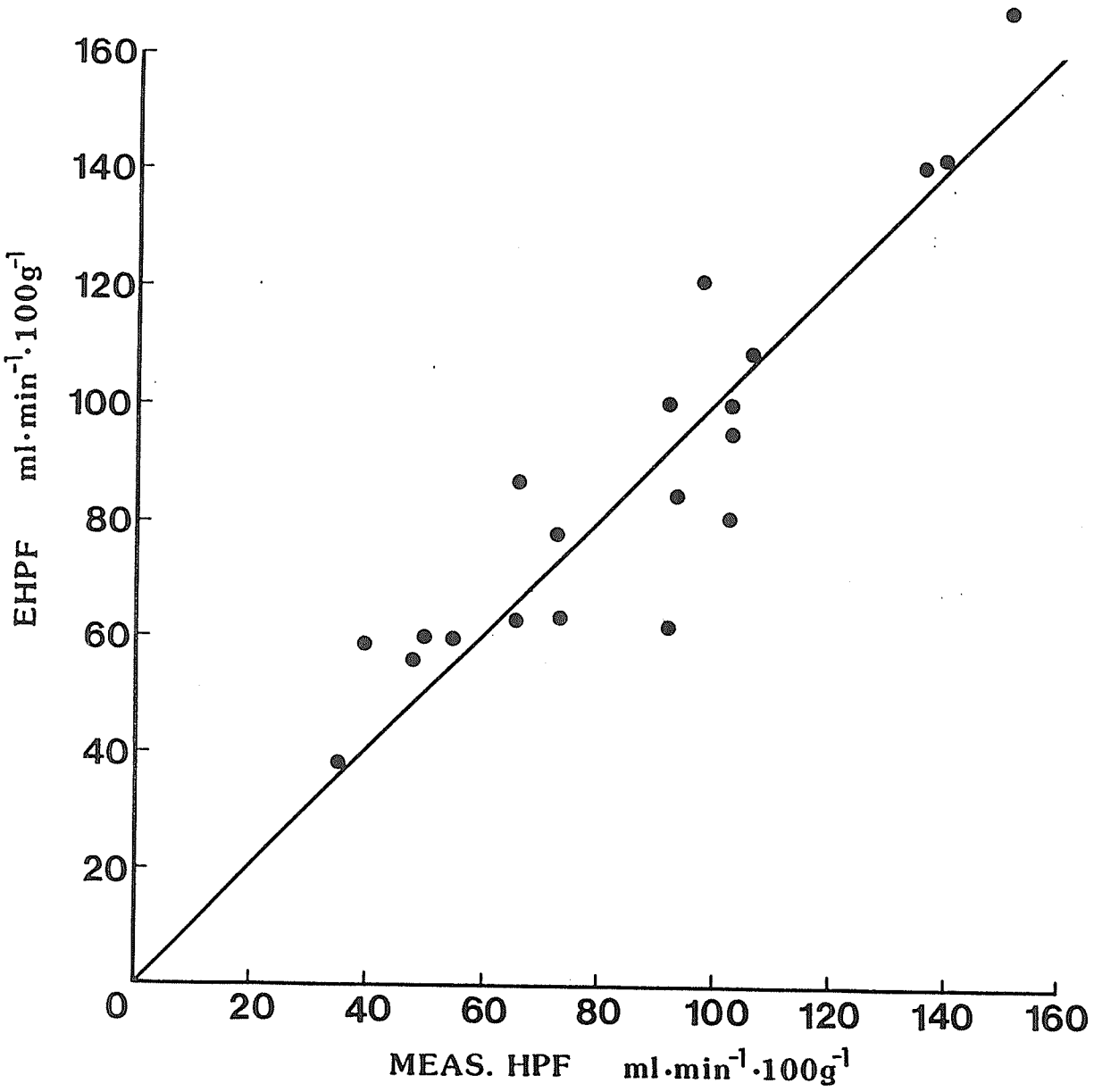

140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹

Fig. 26. Estimated hepatic plasma flows (EHPF) calculated using k (the apparent elimination rate constant) corrected for the extrahepatic uptake rate plotted against directly measured hepatic plasma flows (Meas. HPF) for each of the four doses (second series). The line of identity bisects the two axes.


160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹

Since estimated hepatic plasma flow was not different from the measured plasma flow when the elimination rate constant was corrected for extrahepatic uptake, the use of beta for estimating hepatic plasma flow was investigated. The estimated hepatic plasma flow was found to be similar to the measured flow during the first two series of experiments (figs. 27 and 28). However, the results were also found to be highly variable (figs. 29 and 30). In both series of experiments, estimated flow calculated using beta was not different from that calculated using k (the apparent elimination rate constant corrected for extrahepatic uptake) except during the first bolus dose of the second series ($p=0.03$). When the results were pooled from the first dose of both series, estimated flow was similar to that of the measured flow rate.

D. ICG INTRAVENOUS INFUSIONS

In the fourth series of experiments mean arterial pressures were 109 $\pm$ 11, 145 $\pm$ 18 and 143 $\pm$ 18 mm Hg and hepatic blood flows were 137 $\pm$ 30, 133 $\pm$ 34 and 130 $\pm$ 33 ml/min/100g liver at the beginning, end of the first infusion and end of the second infusion respectively. Mean liver weight was 19 $\pm$ 1 g/kg body weight. Mean hematocrit was 41 $\pm$ 3% and this did not change by more than 1%

Fig. 27. Hepatic plasma flows measured directly and calculated using beta for each of the three doses (first series). Mean $\pm$ S.E. N = 5.

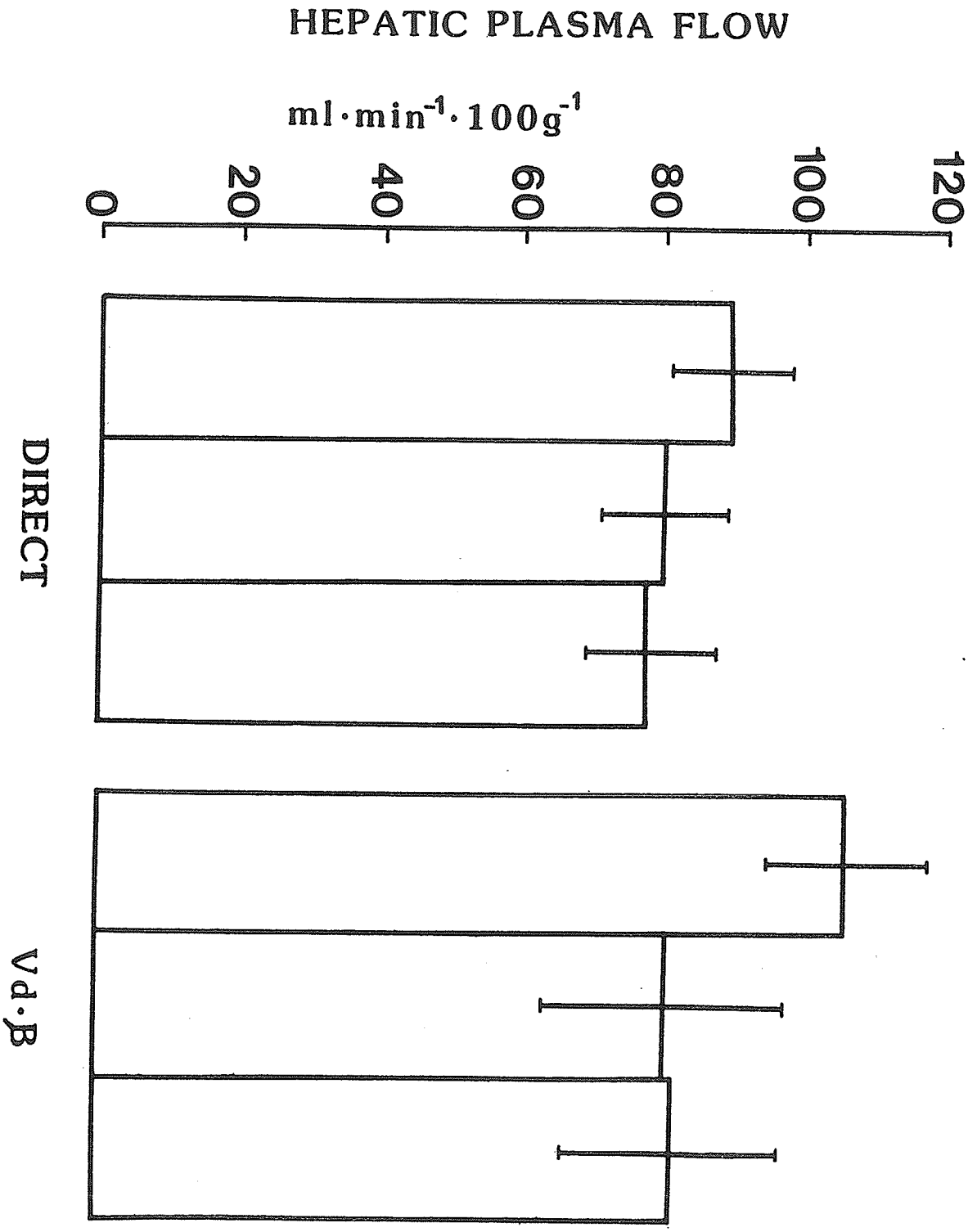

HEPATIC PLASMA FLOW
ml·min-1·100g-1
0
20
40
60
80
100
120
DIRECT
Vd.β

Fig. 28. Hepatic plasma flows mesured directly and calculated using beta for each of the four doses (second series). Mean $\pm$ S.E. N = 5.

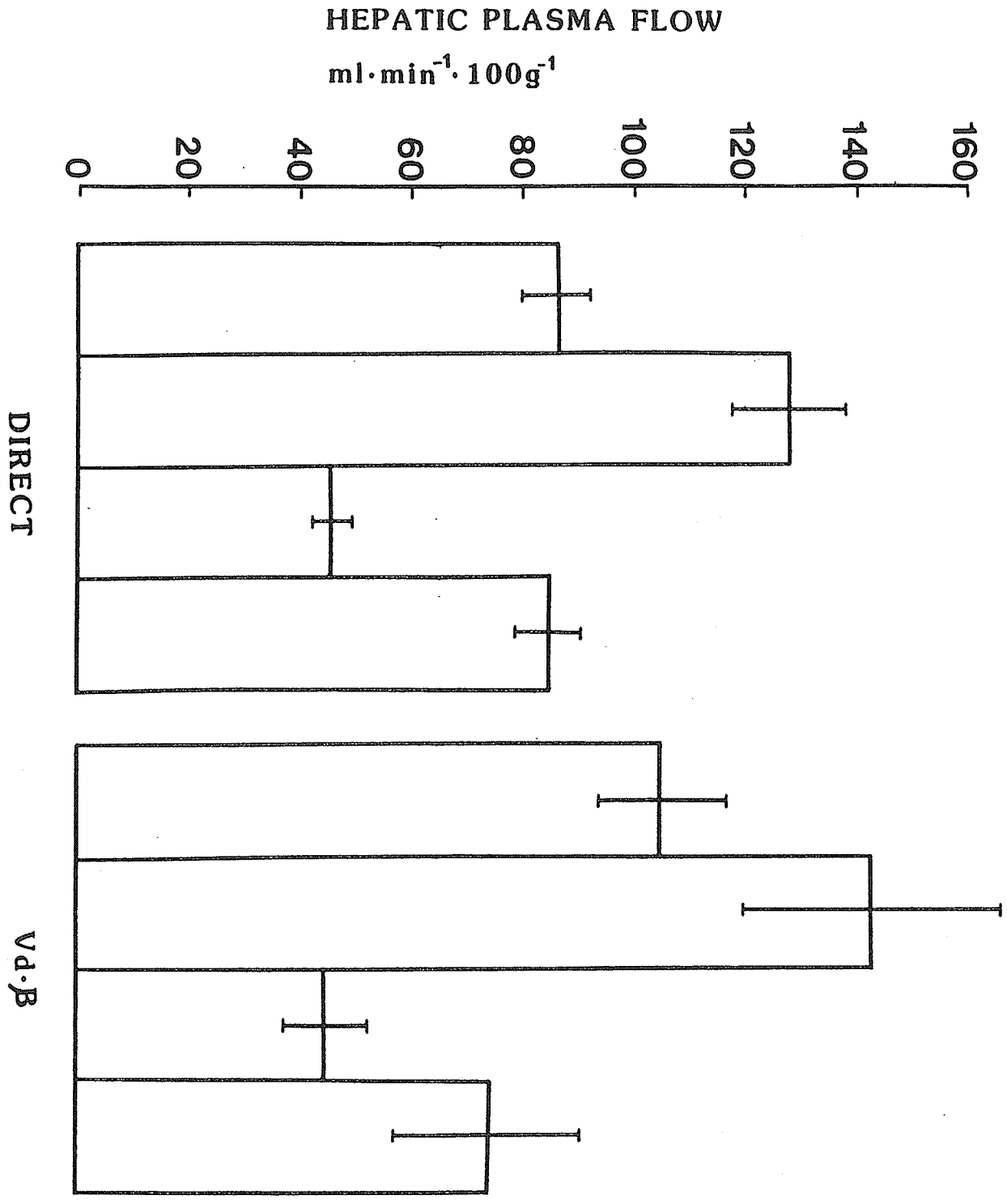

HEPATIC PLASMA FLOW
ml·min-1·100g-1
0
20
40
60
80
100
120
140
160
DIRECT
Vd.β

Fig. 29. Estimated hepatic plasma flows (EHPF) calculated by using beta plotted against the directly measured flows (Meas. HPF) for each of the three doses (series one). Individual values for the 5 cats are shown by the 5 symbols.

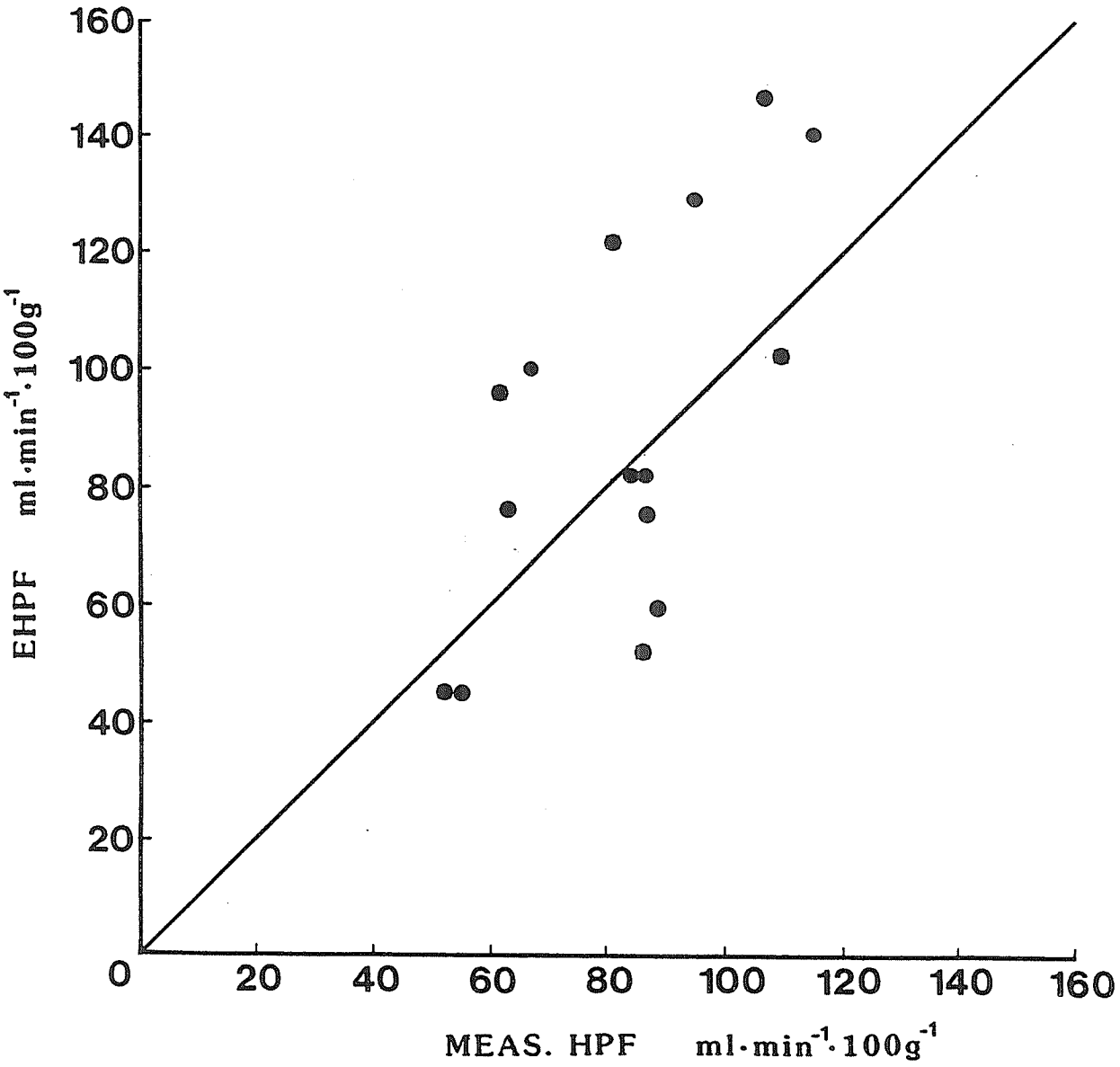
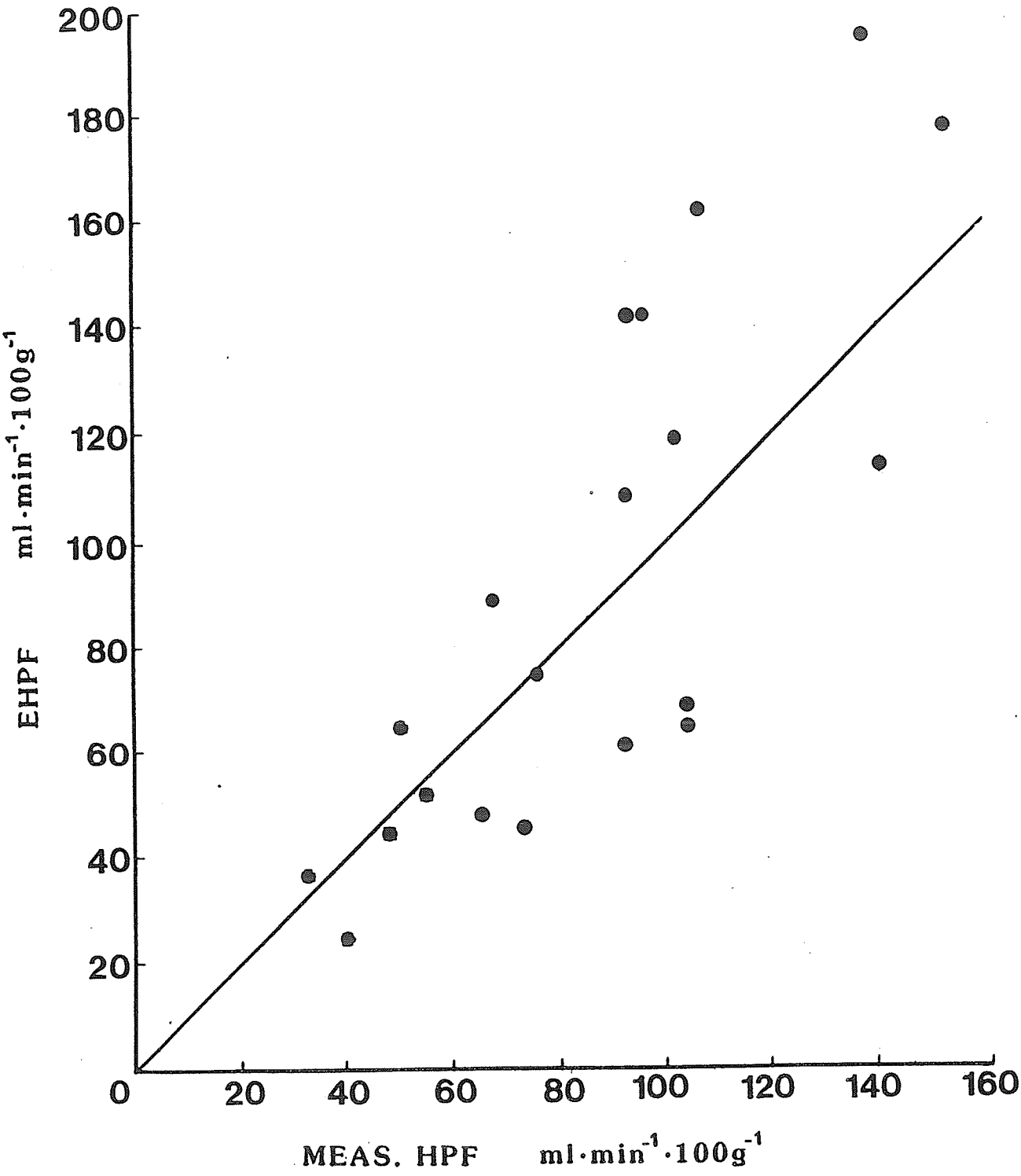

160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹

Fig. 30. Estimated hepatic plasma flows (EHPF) calculated by using beta plotted against the directly measured flows (Meas. HPF) for each of the four doses (series two). Individual values for the 5 cats are shown by the 5 symbols.


200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹

throughout each experiment.

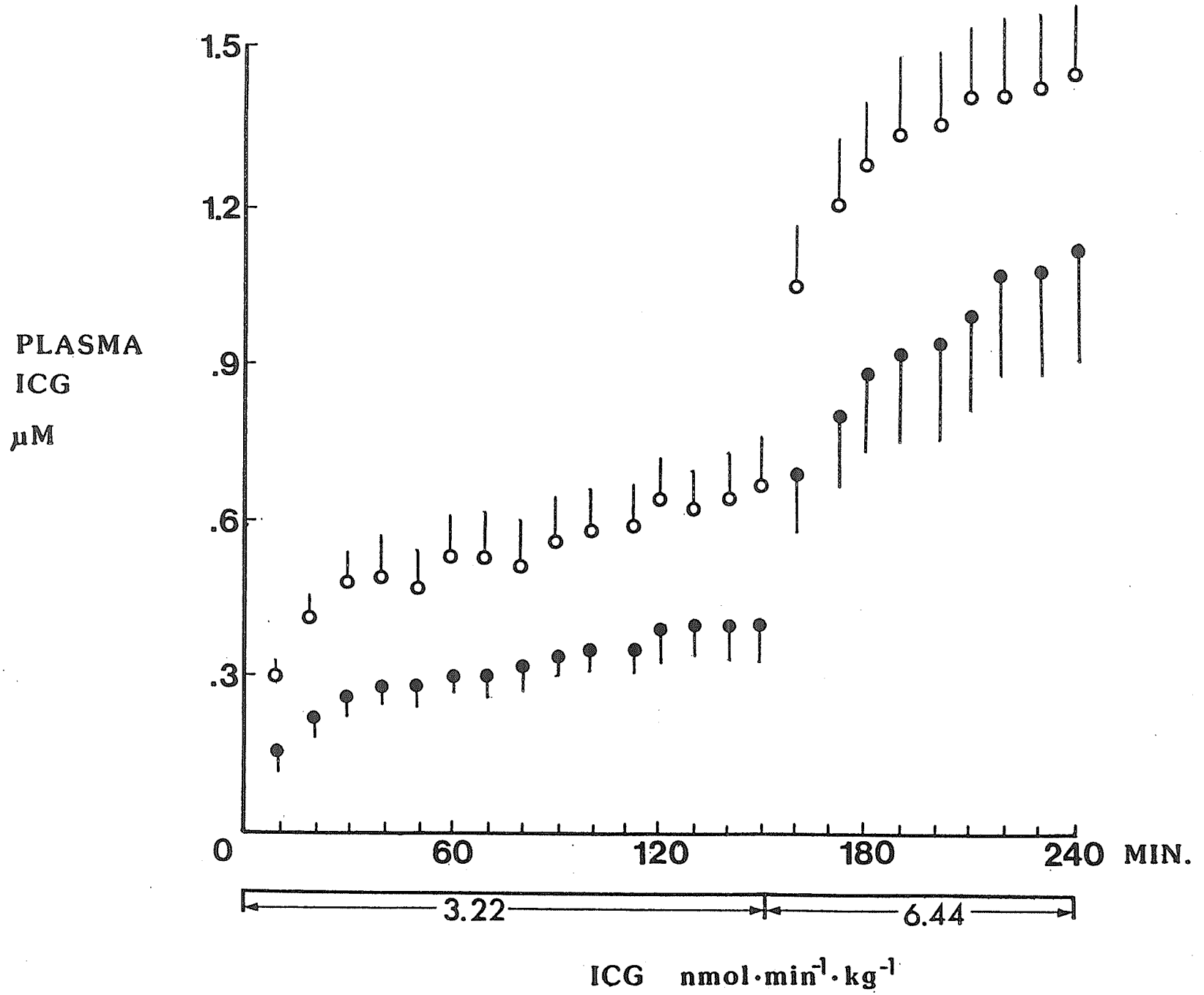
Mean arterial pressures during the fifth series were 87 ± 7 and 95 ± 7 mm Hg at the start and end of the control flow, 127 ± 3 and 138 ± 3 mm Hg at the start and end of the raised flow and 80 ± 10 and 85 ± 10 mm Hg at the start and end of the reduced flow period. Hepatic blood flows were 120 ± 8 ml/min/100g liver during the control period, 190 ± 12 ml/min/100g liver during the raised flow and 64 ± 4 ml/min/100g liver during the low flow period. Mean liver weight was 21 ± 2.1 g/kg body weight. Mean hematocrit was 35 ± 1% and did not change by more than 1% throughout the experiments.

The total amount of ICG in liver and bile samples was 87% of that administered at the end of the second series of experiments which was terminated 4 h after the administration of the first ICG dose.

1. PLASMA ICG MEASUREMENTS AND EXTRACTIONS

Arterial and hepatic venous plasma ICG concentrations during the two infusions of ICG (fourth series) are presented in fig. 31. Both arterial and hepatic venous concentrations increased throughout the 150 min low dose infusion and 90 min high dose infusion. When the infusion

Fig. 31. Arterial (○) and hepatic venous (●) plasma ICG levels during infusion of ICG at two rates. Means ± S.E. for 4 cats.


PLASMA
ICG
µM
1.5
1.2
.9
.6
.3
0
60
120
180
240 MIN.
3.22
6.44
ICG nmol·min⁻¹·kg⁻¹

rate was doubled, arterial and hepatic venous concentrations increased more rapidly until the infusion was stopped at 240 min. Even at this minimal infusion rate, steady state plasma concentrations could not be achieved.

Plasma arterial and hepatic venous ICG concentrations for the fifth series of altered flow experiments are shown in fig. 32. Arterial and hepatic venous concentrations tended to rise during the period of control flow but the change was not statistically significant over the time period. When flow was increased, arterial concentrations fell slightly while hepatic venous concentrations remained unchanged. When flow was decreased, both arterial and hepatic venous concentrations continuously increased until the end of the 60 min period.

Extraction of ICG, calculated as the arteriovenous difference divided by the arterial plasma concentration [(Ca-Cv)/Ca] for each paired arterial and hepatic venous plasma samples, is shown in fig. 33 for the fourth series of experiments. There was a small but significant decrease in extraction with time. This was greater at the higher dose than at the lower dose. Initial extraction was 0.47 $\pm$ 0.08 Mean extraction was calculated for 40-90 min into each infusion period to facilitate comparison with the fifth series of experiments. Mean extraction was 0.42 $\pm$

Fig. 32. Arterial (O) and venous (●) plasma ICG concentrations during constant infusion of ICG at 3 different flows. Mean ± S.E. for 5 cats.

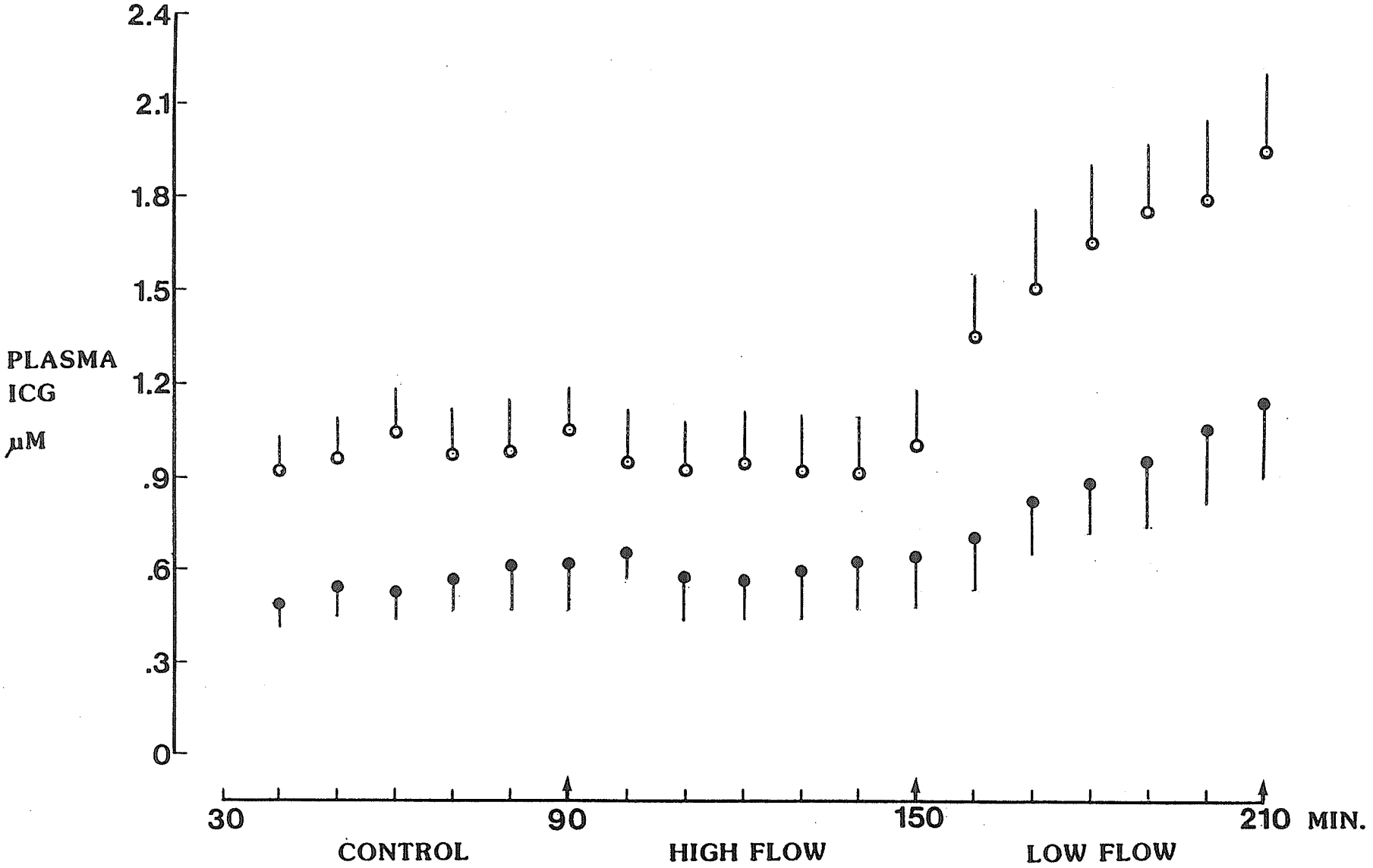
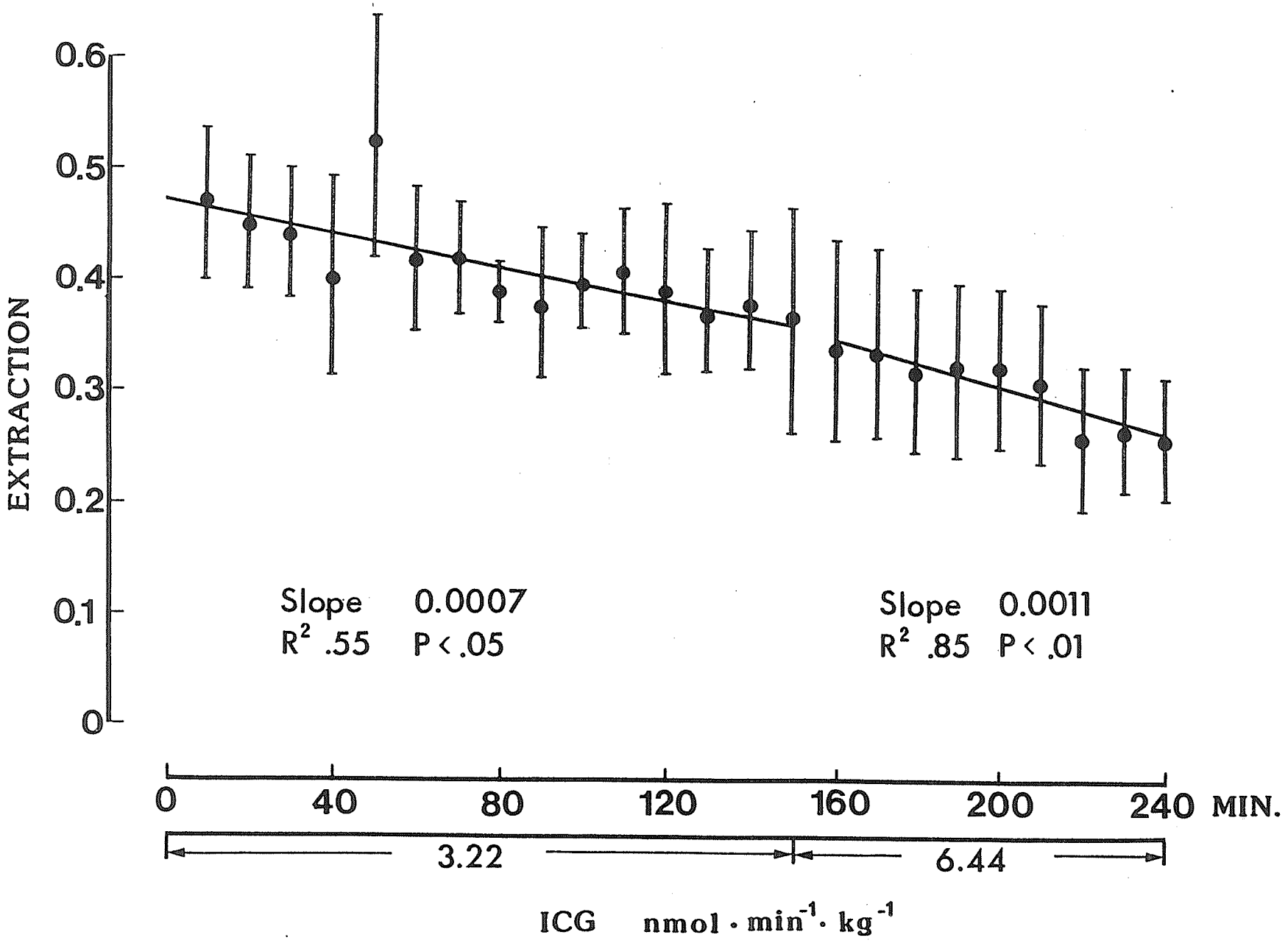

2.4
2.1
1.8
1.5
1.2
.9
.6
.3
0
PLASMA
ICG
µM
30
90
150
210
MIN.
CONTROL
HIGH FLOW
LOW FLOW

Fig. 33. Extraction of indocyanine green plotted against time for the two infusions (series four). Mean $\pm$ S.E. N = 4.


EXTRACTION
0.6
0.5
0.4
0.3
0.2
0.1
0
Slope 0.0007
R² .55 P < .05
Slope 0.0011
R² .85 P < .01
0
40
80
120
160
200
240 MIN.
3.22
6.44
ICG nmol · min⁻¹ · kg⁻¹

0.06 for the first dose and decreased to 0.28 $\pm$ 0.07 for the second dose ($p<0.05$). As in the first series of experiments, the decrease in extraction correlated with the amount of ICG that had been accumulated by the liver ($r = -0.75$, $p<0.01$; fig. 34).

In series 5 within each flow period, extraction calculated for the individual data points did not change significantly but the standard errors were large (fig. 35). The standard errors reflected variability in extraction between animals. When the data for each flow period were analysed by blocked ANOVA, extraction was 0.43, 0.37 and 0.46 during control, raised flow and low flow periods, respectively with a pooled S.E. of 0.01. These values were significantly different ($P<0.05$, Duncan's multiple range test).

2. SYSTEMIC ICG CLEARANCE

Systemic clearance of ICG was calculated as infusion rate divided by arterial concentration at each time and the results are shown in fig. 36 for the fourth series of experiments. These values are the estimated values for hepatic plasma flow if extraction is assumed to be 100%. Like extraction, clearance showed a steady decline with time. Mean clearance was calculated for 40-90 min into

Fig. 34. Extraction of indocyanine green plotted against amount remaining to be excreted (N = 4). Individual animals are represented by the different symbols.

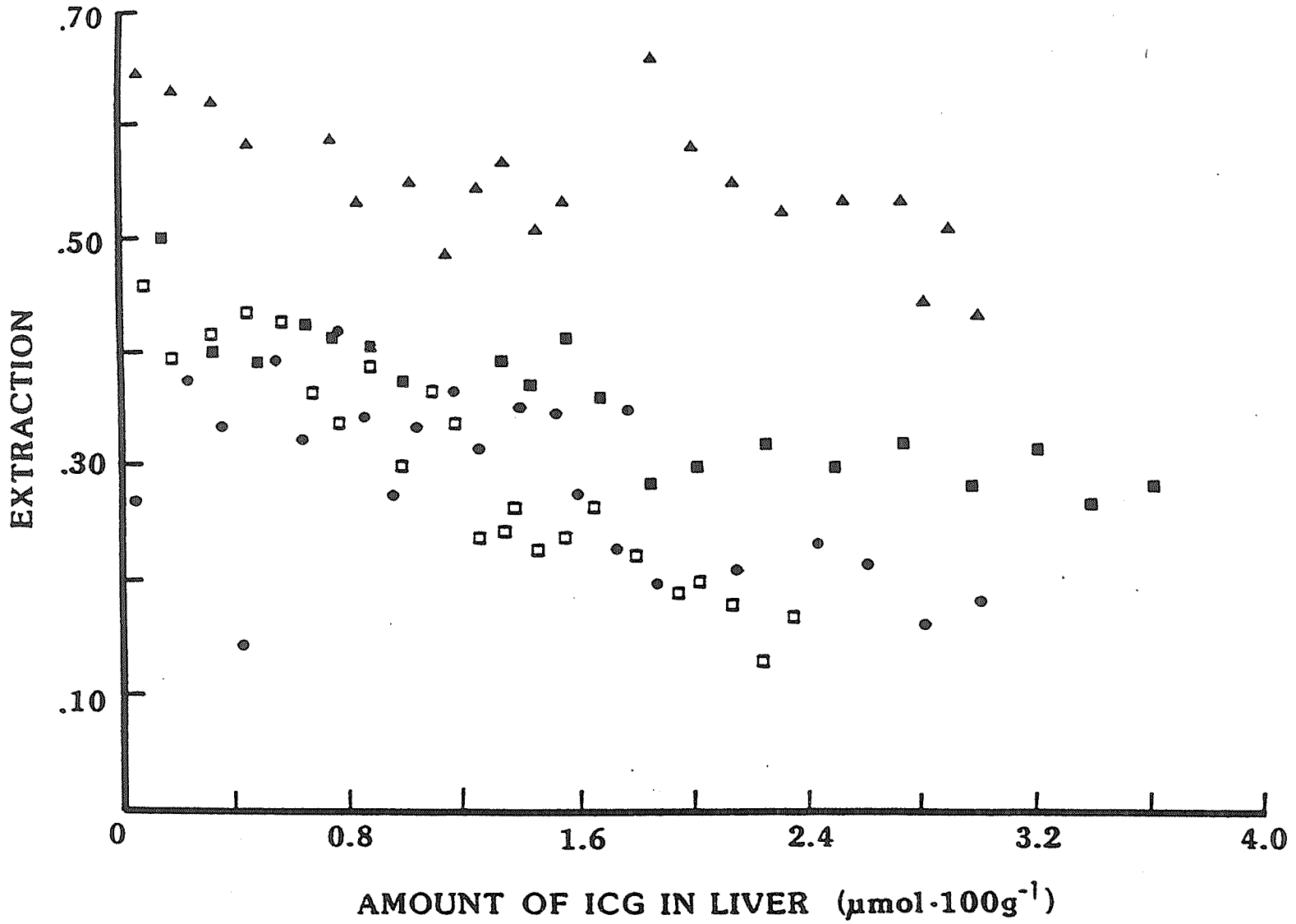

.70
.50
.30
.10
EXTRACTION
0
0.8
1.6
2.4
3.2
4.0
AMOUNT OF ICG IN LIVER (μmol·100g⁻¹)

Fig. 35. Extraction of indocyanine green plotted against time for the altered flow experiments (series five). Mean $\pm$ S.E. N = 5.

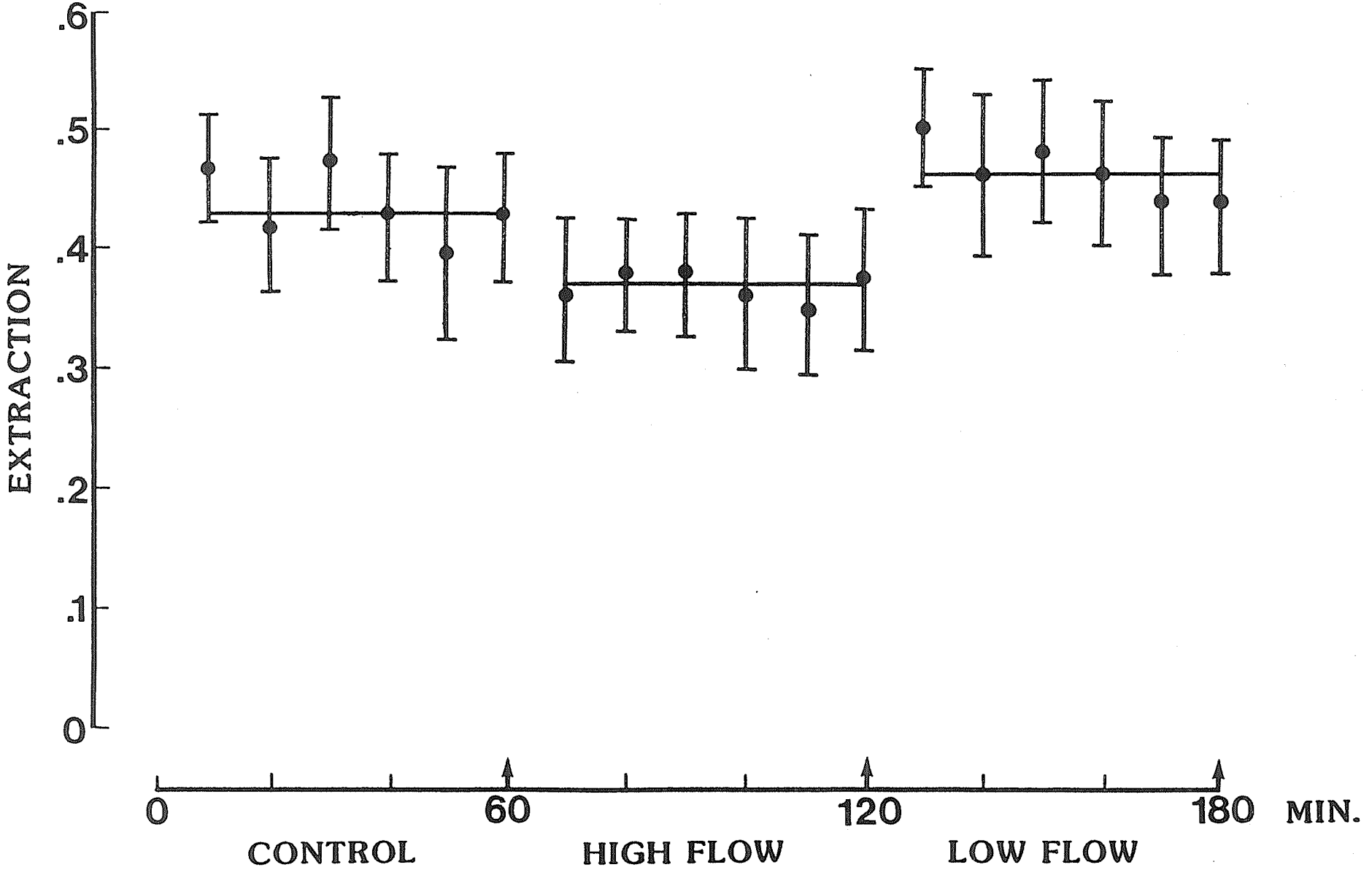
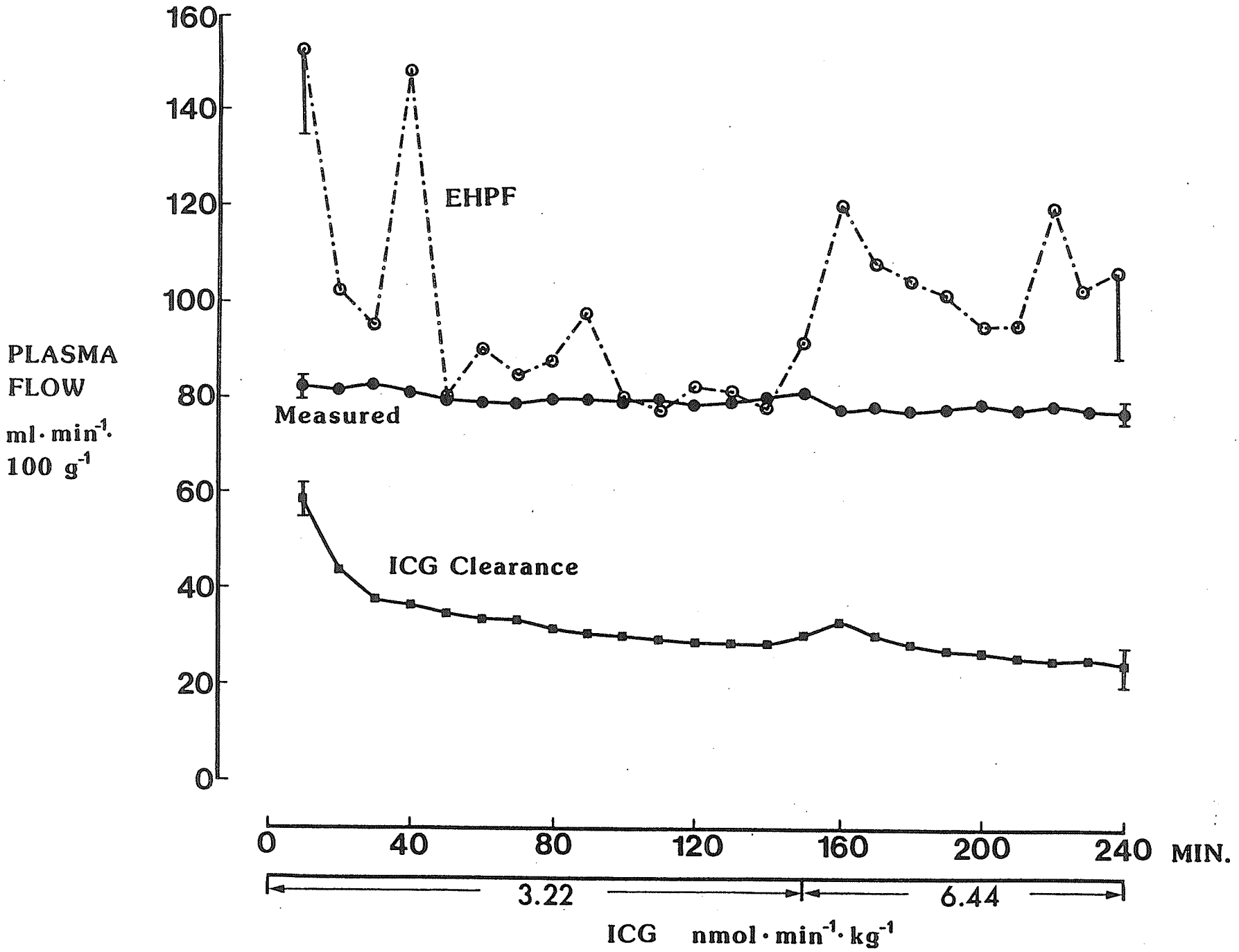

.6
.5
.4
.3
.2
.1
0
EXTRACTION
0
60
120
180
MIN.
CONTROL
HIGH FLOW
LOW FLOW

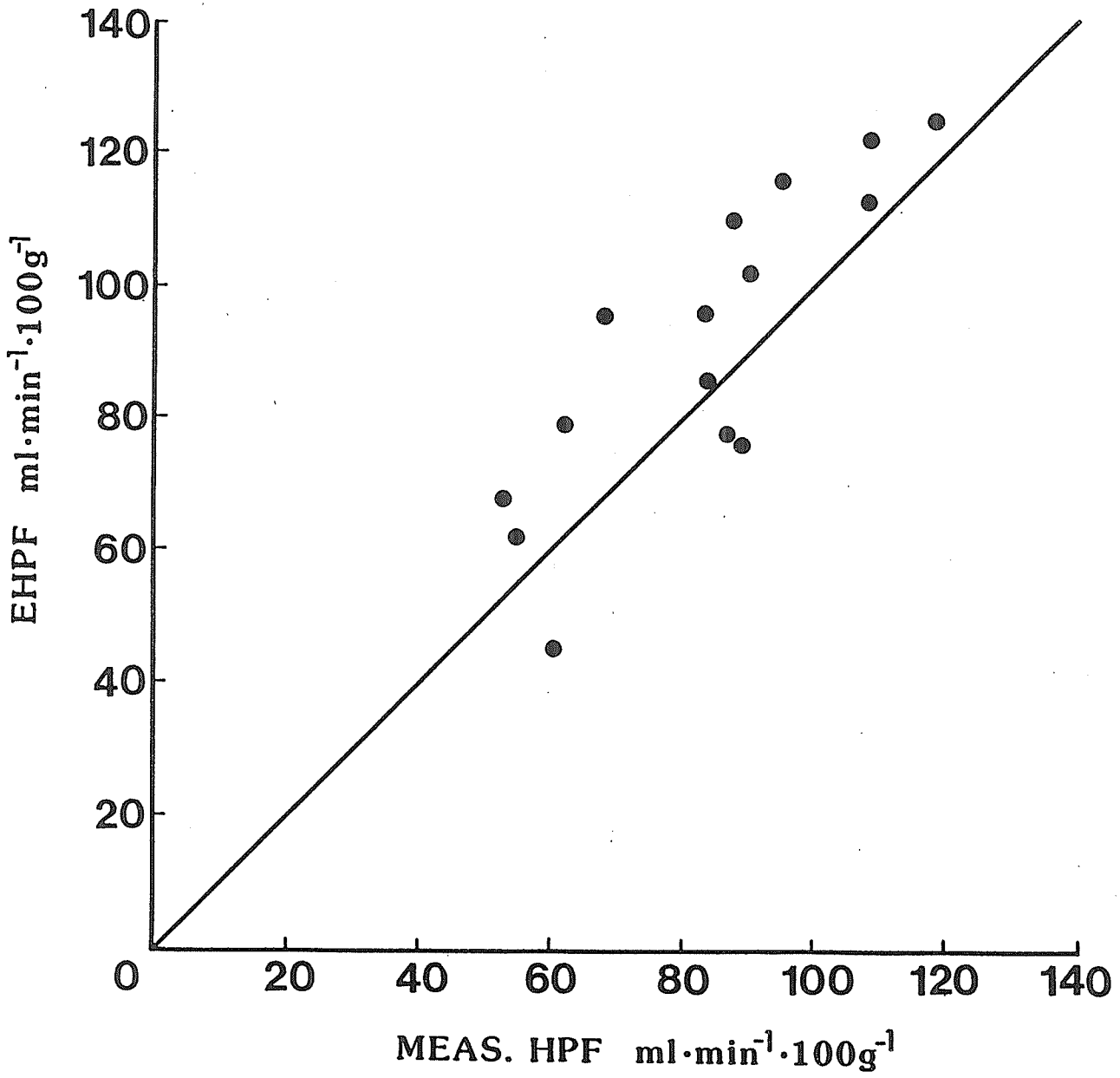
Fig. 36. Measured plasma flows, calculated ICG clearances and estimated hepatic plasma flows (EHPF) for each time period during the infusions. Means for 4 cats with the pooled S.E. from blocked analysis of variance to remove variability among cats. In spite of elimination of this source of variability, estimated hepatic plasma flows show marked variability.

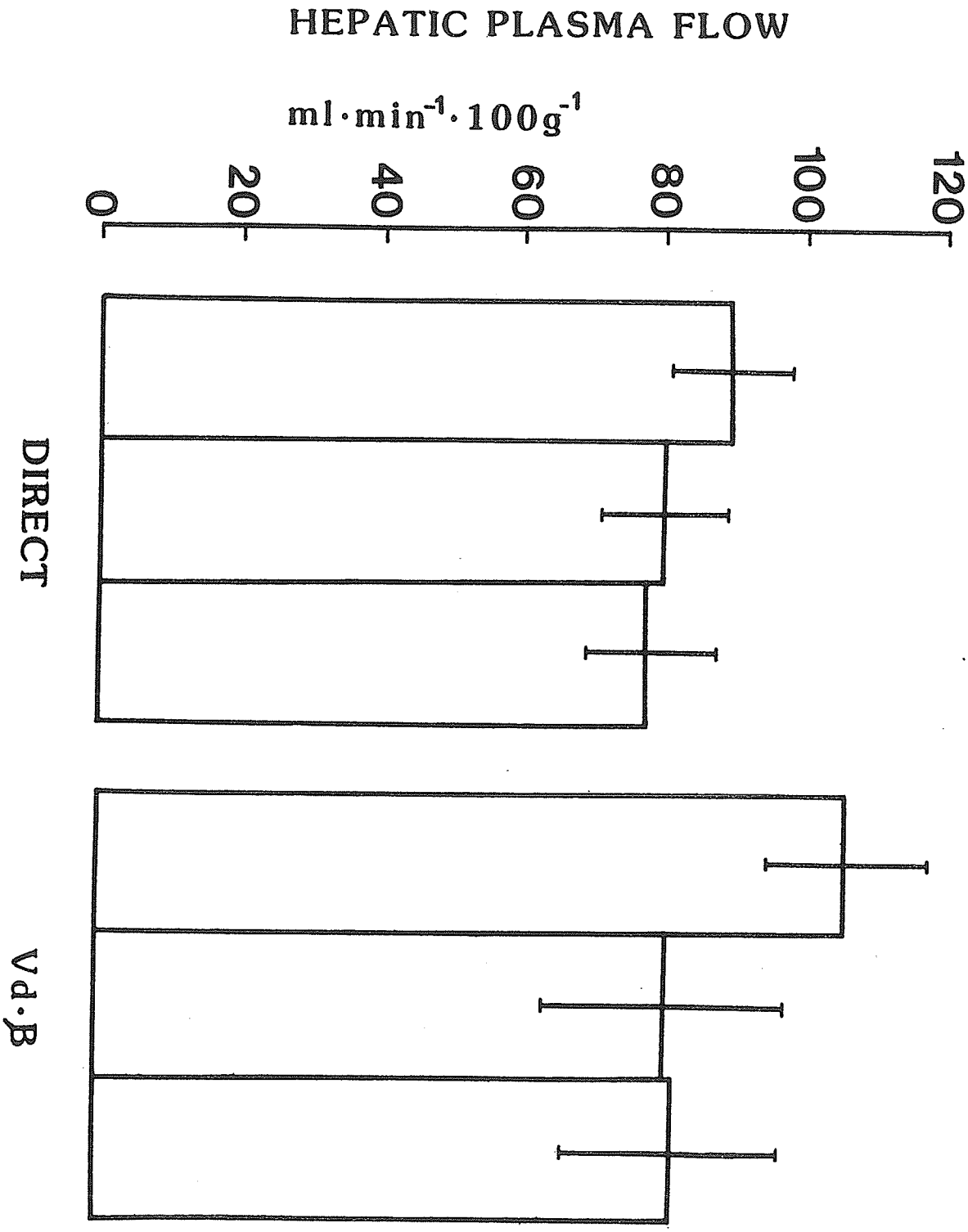

160
140
120
100
80
60
40
20
0
PLASMA
FLOW
ml · min⁻¹ ·
100 g⁻¹
EHPF
Measured
ICG Clearance
0
40
80
120
160
200
240
MIN.
3.22
6.44
ICG nmol · min⁻¹ · kg⁻¹

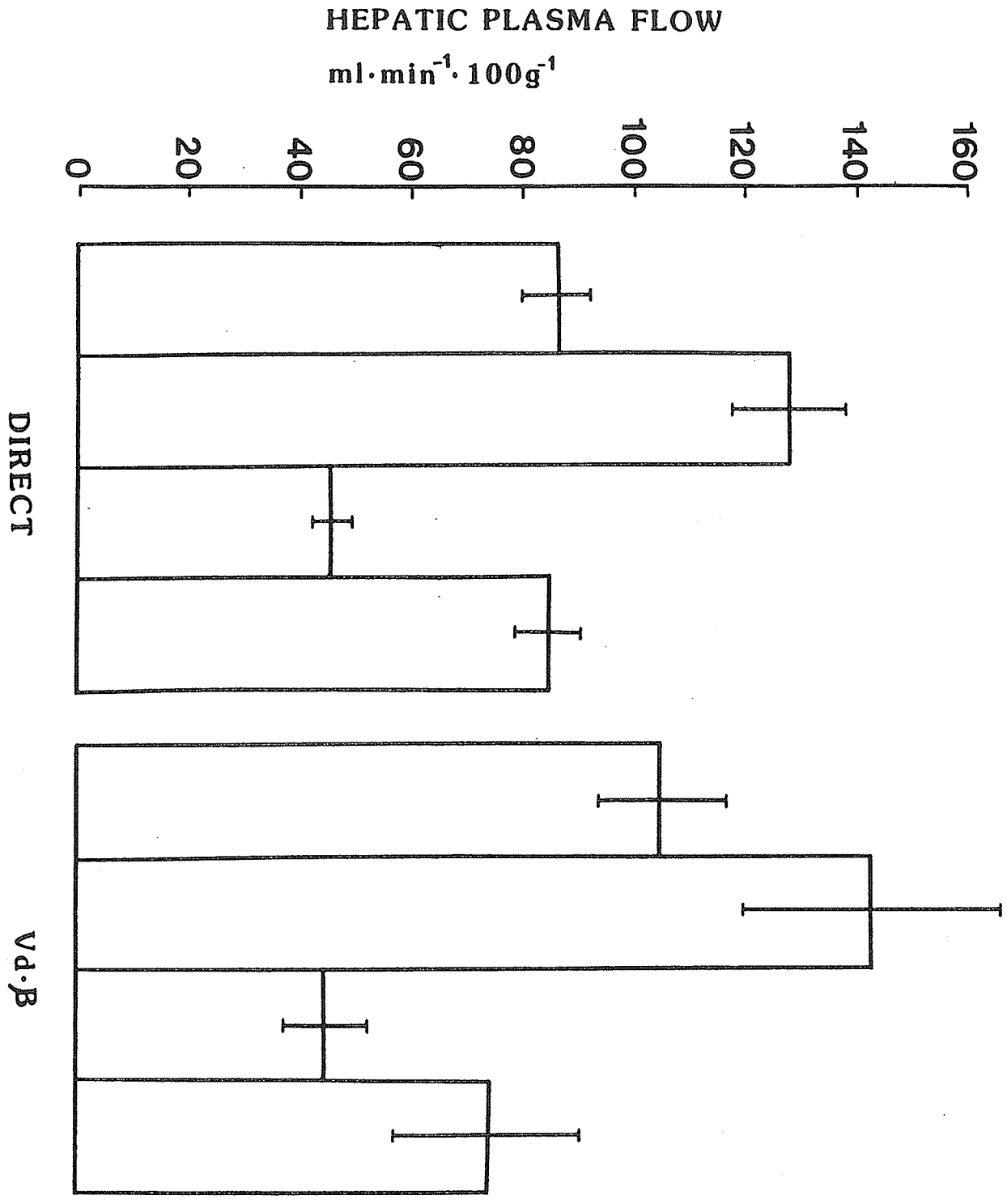
each infusion period. Mean systemic clearance was 35 ± 6 ml/min/100g liver for the first dose and 25 ± 3 ml/min/100g liver for the second dose. This difference approached statistical significance (P = 0.11). A theoretically better estimate of hepatic plasma flow was calculated by dividing clearance by extraction at each time and these results are also shown in fig. 36. Initially, mean estimated hepatic plasma flow exceeded measured plasma flow at both infusion rates. During the latter portion of the first infusion period estimated hepatic plasma flow was not different from the measured flow rate. Mean estimates of hepatic plasma flow were calculated for 40-90 min into each infusion period. Mean estimated plasma flow was 99 ± 15 ml/min/100g liver for the first dose and 103 ± 9 ml/min/100g liver for the second dose. Measured plasma flow remained constant at 80 ml/min/100g liver. Mean hepatic clearance calculated as uptake divided by arterial plasma concentration was 29 ± 5 and 20 ± 5 ml/min/100g liver for the low and high doses 40-90 min into each infusion, respectively. These values, although lower, were not different from the systemic clearances calculated as infusion rate divided by mean arterial plasma concentrations.

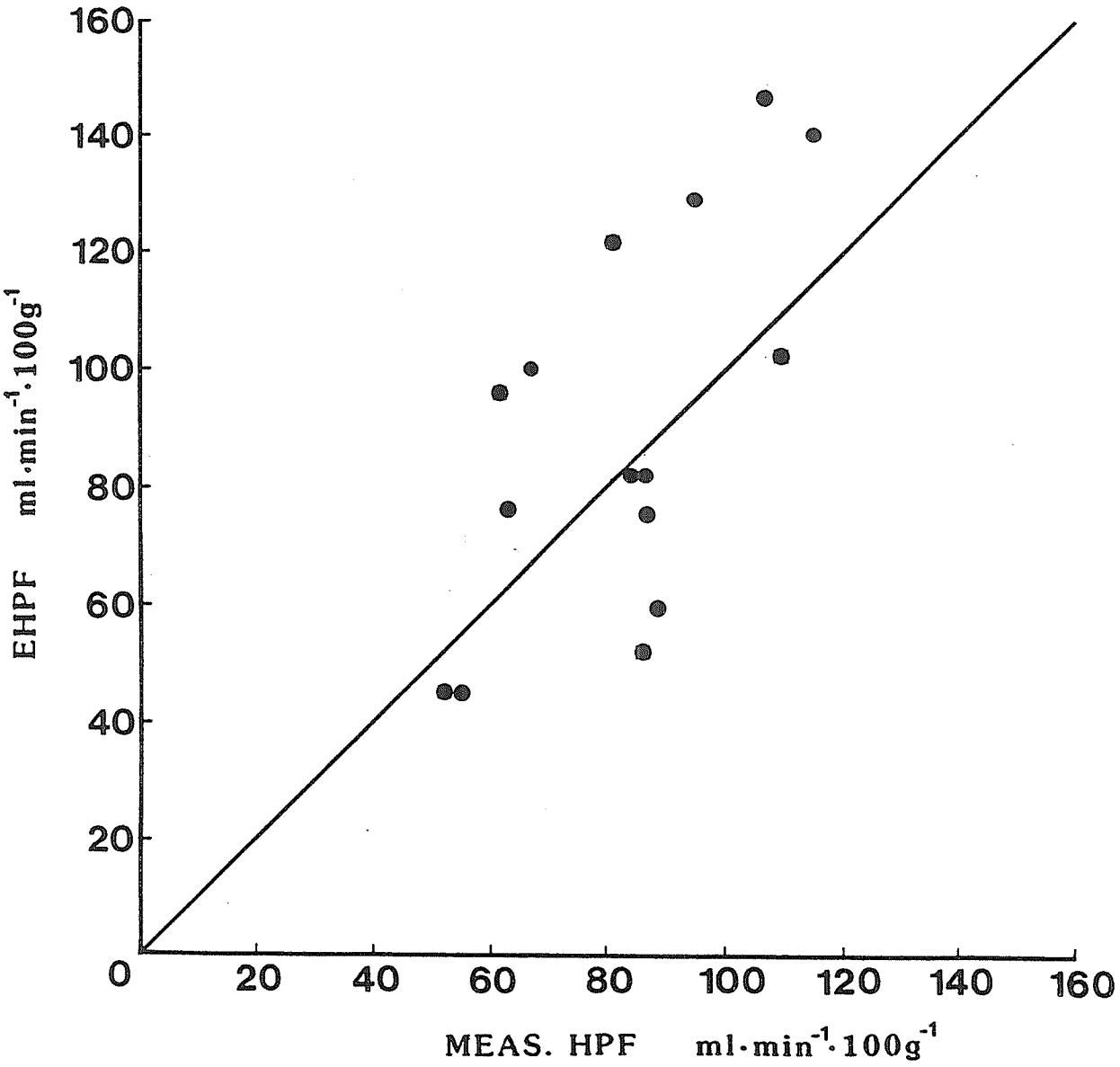
Systemic clearances, measured plasma flows and estimated plasma flows are shown in fig. 37 for the altered flow experiments. In this series the initial 30 min

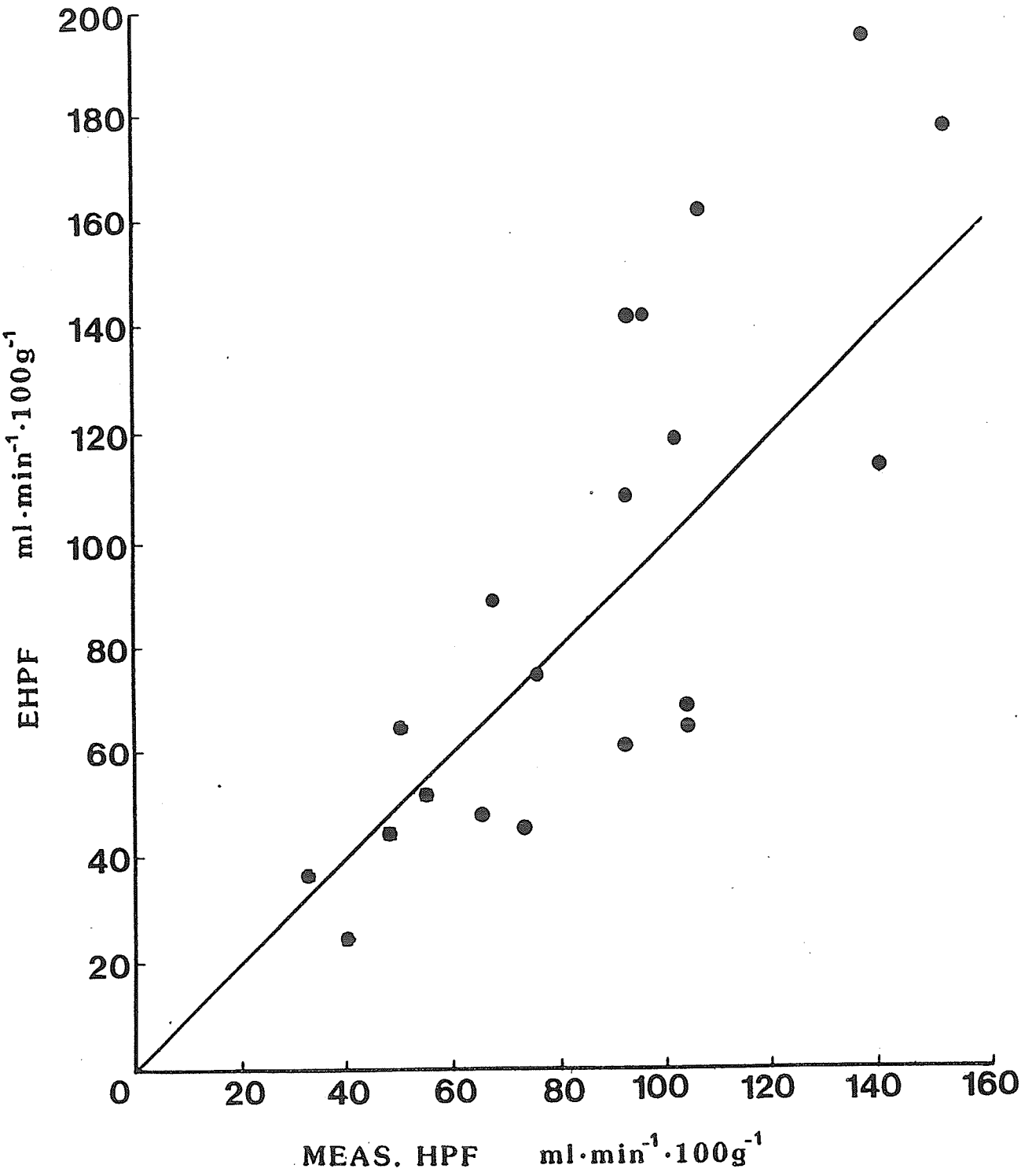
Fig. 37. Measured plasma flow (M), calculated ICG clearance (C) and estimated hepatic plasma flow (E) for each time period during constant infusion of ICG at 3 different flows. The graph shows means for 5 cats with the pooled S.E. from blocked ANOVA to remove variability among cats.

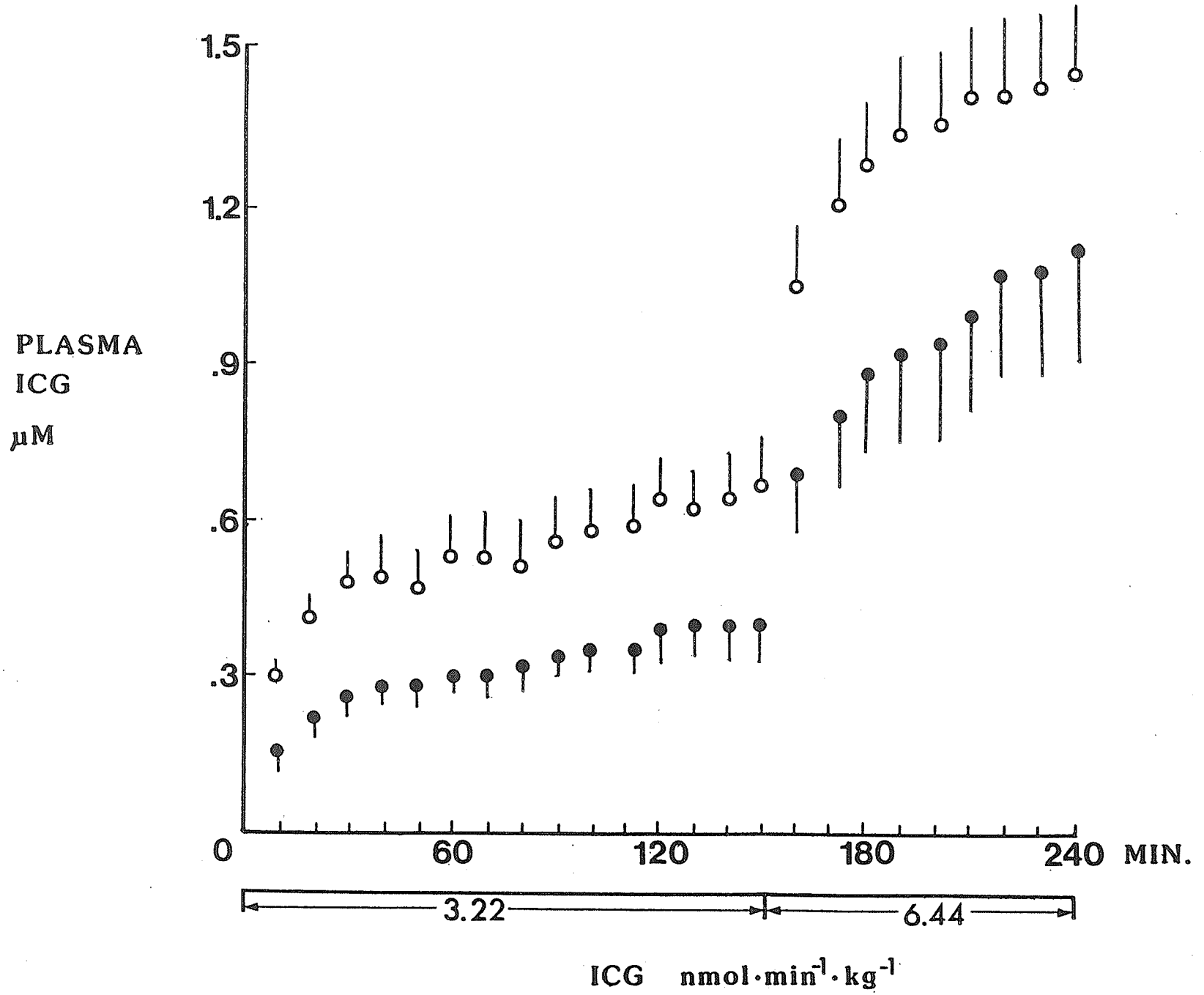

140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹

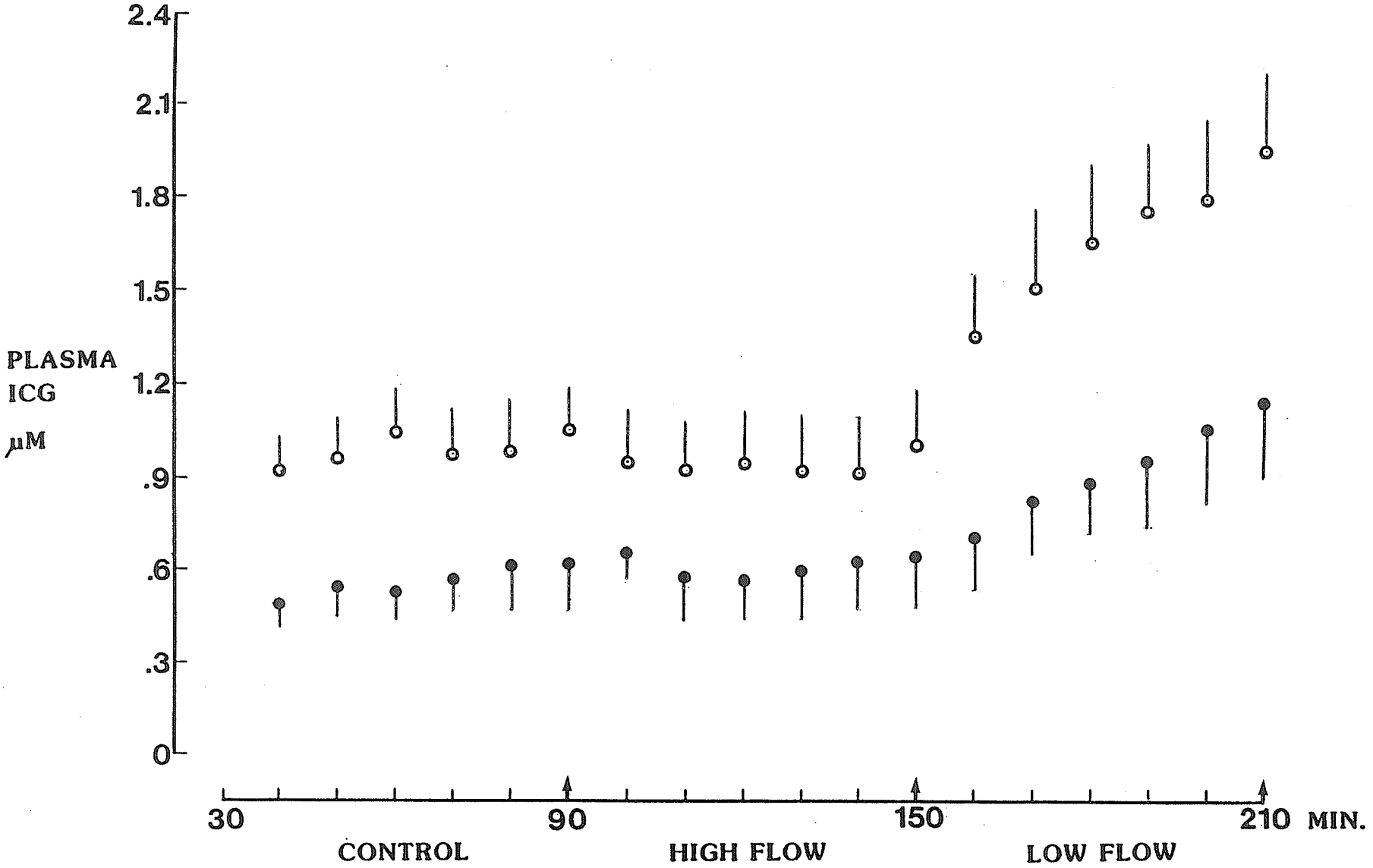

HEPATIC PLASMA FLOW
ml·min-1·100g-1
0
20
40
60
80
100
120
DIRECT
Vd.β

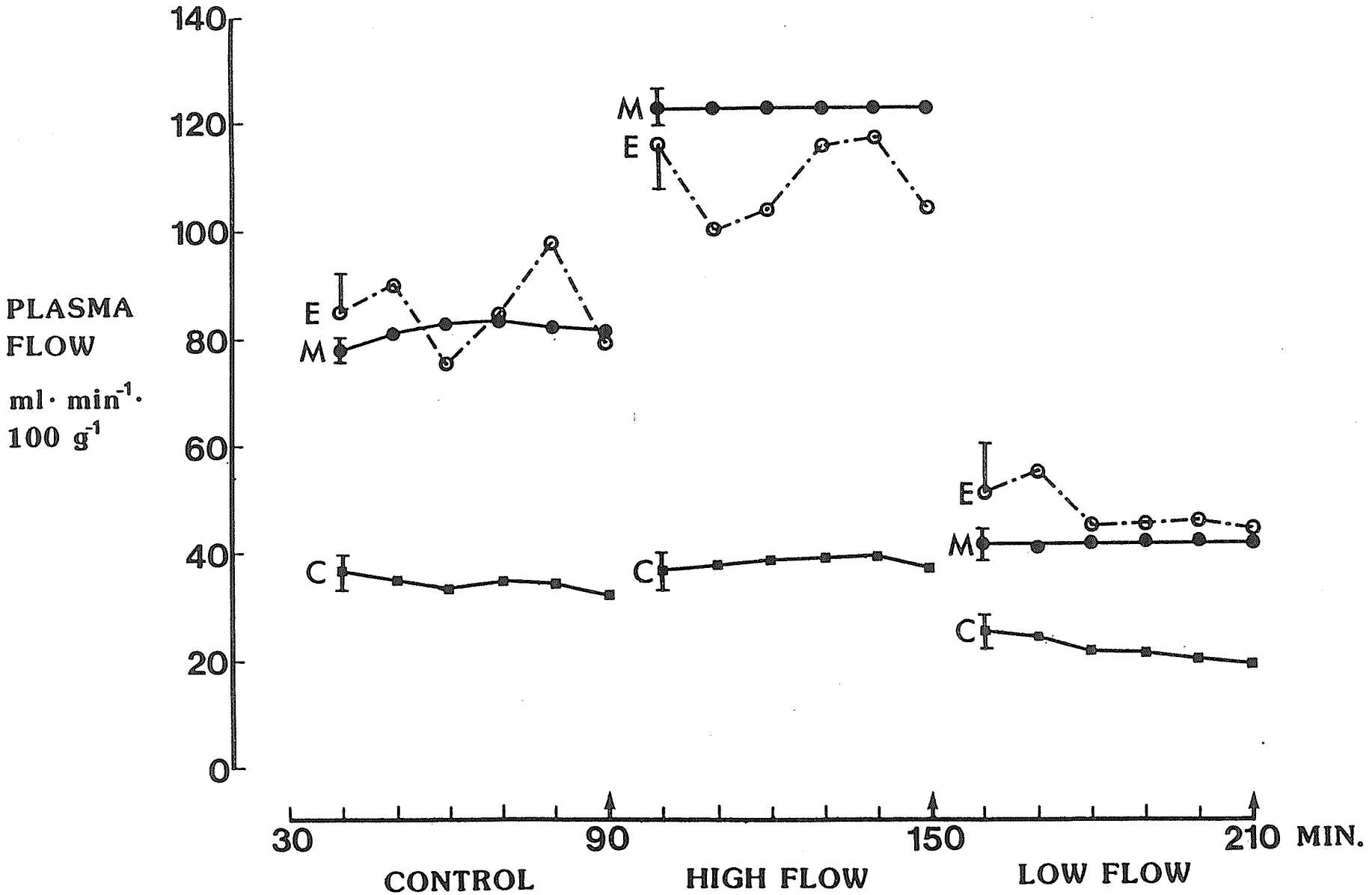

HEPATIC PLASMA FLOW
ml·min-1·100g-1
0
20
40
60
80
100
120
140
160
DIRECT
Vd.β

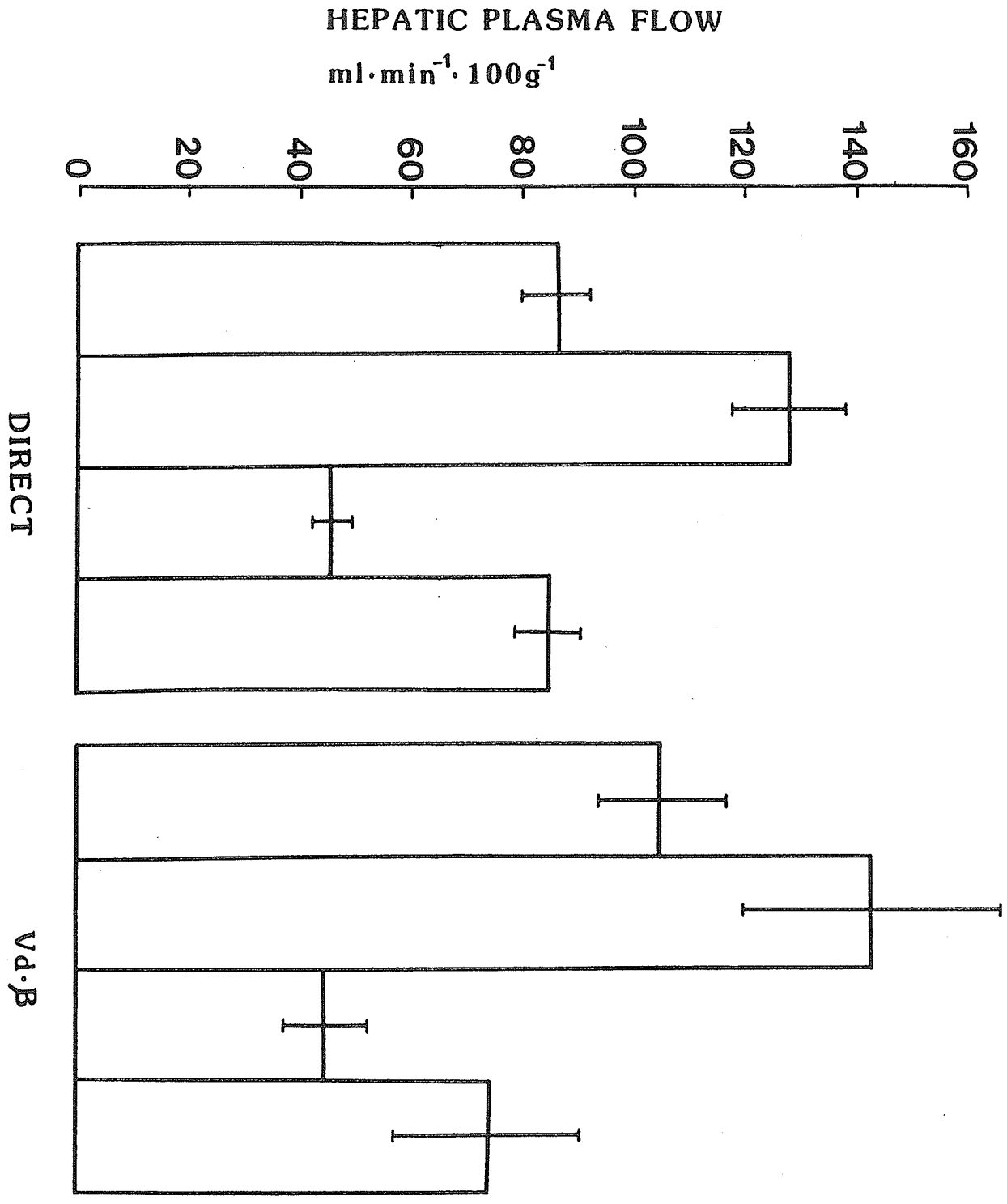

160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹

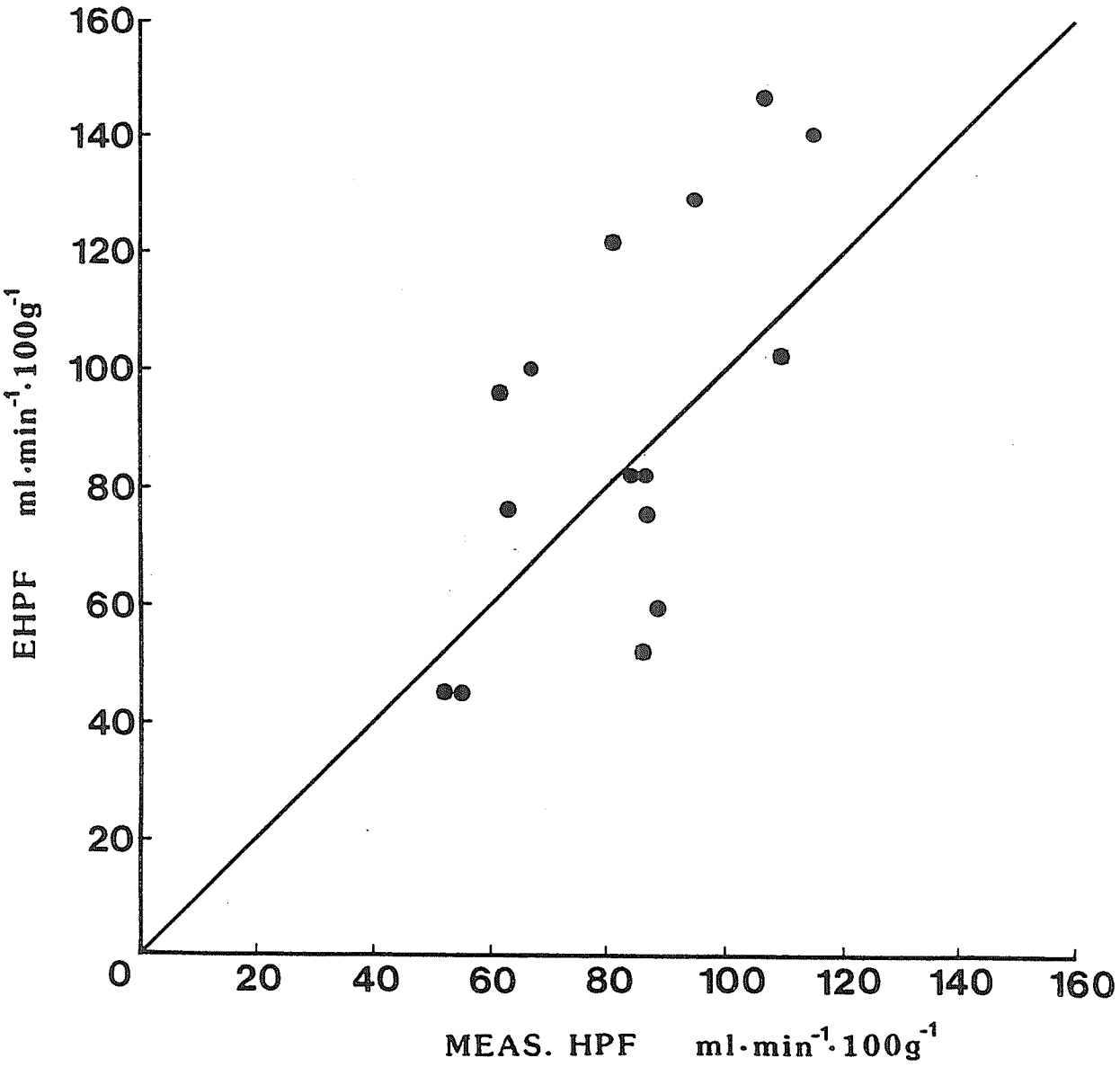

200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹

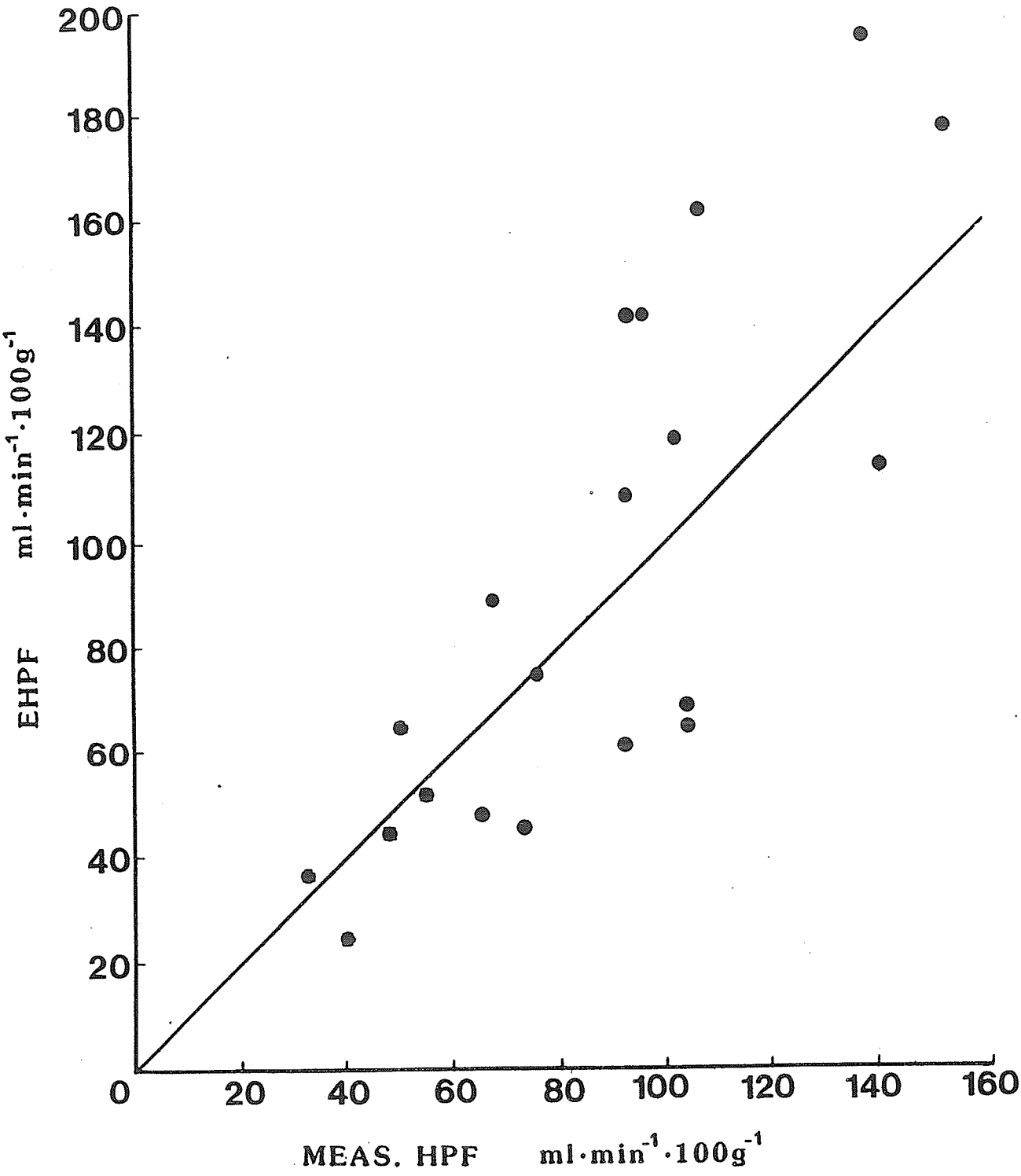

PLASMA
ICG
µM
1.5
1.2
.9
.6
.3
0
60
120
180
240 MIN.
3.22
6.44
ICG nmol·min⁻¹·kg⁻¹

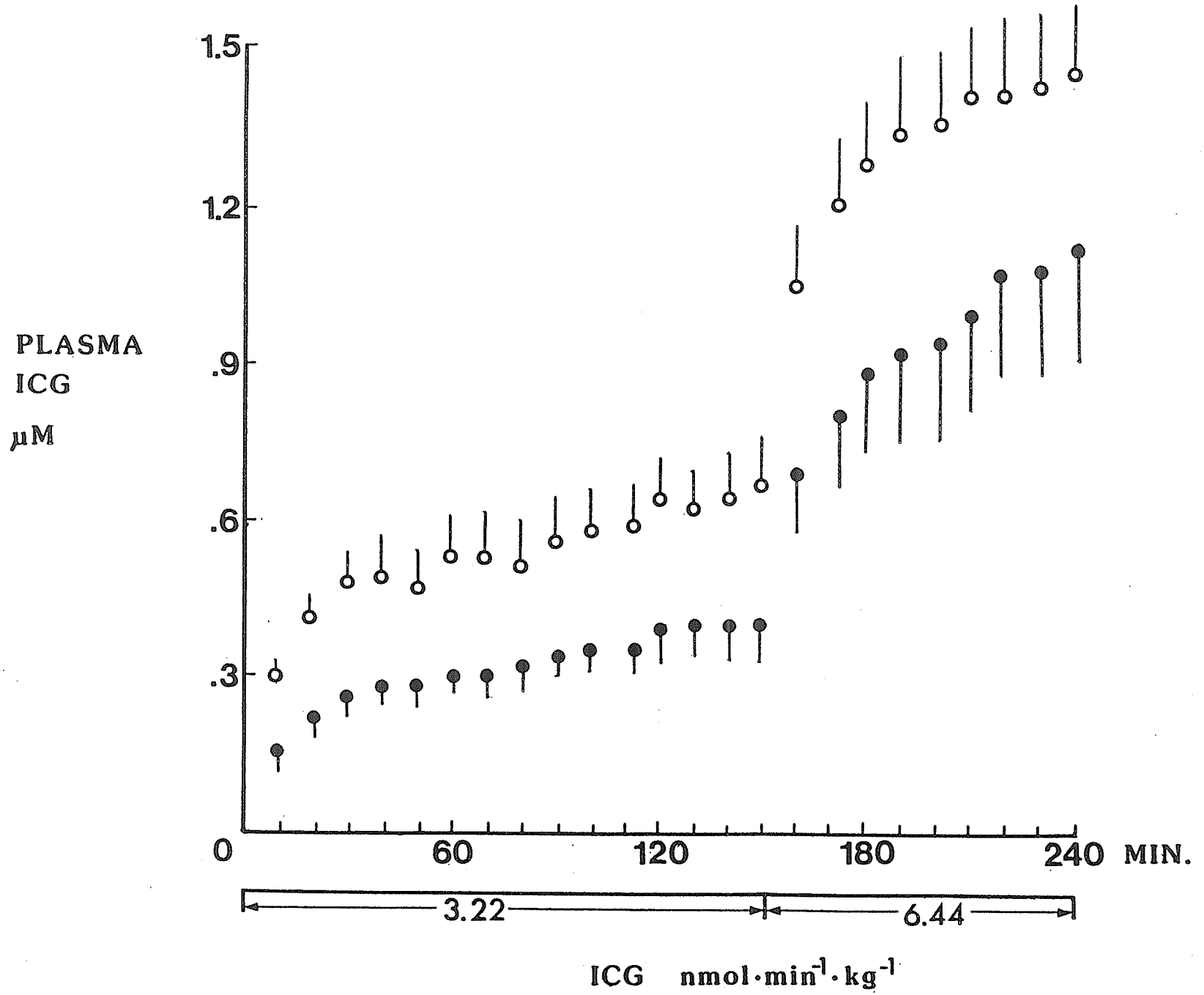

2.4
2.1
1.8
1.5
1.2
.9
.6
.3
0
PLASMA
ICG
µM
30
90
150
210
MIN.
CONTROL
HIGH FLOW
LOW FLOW

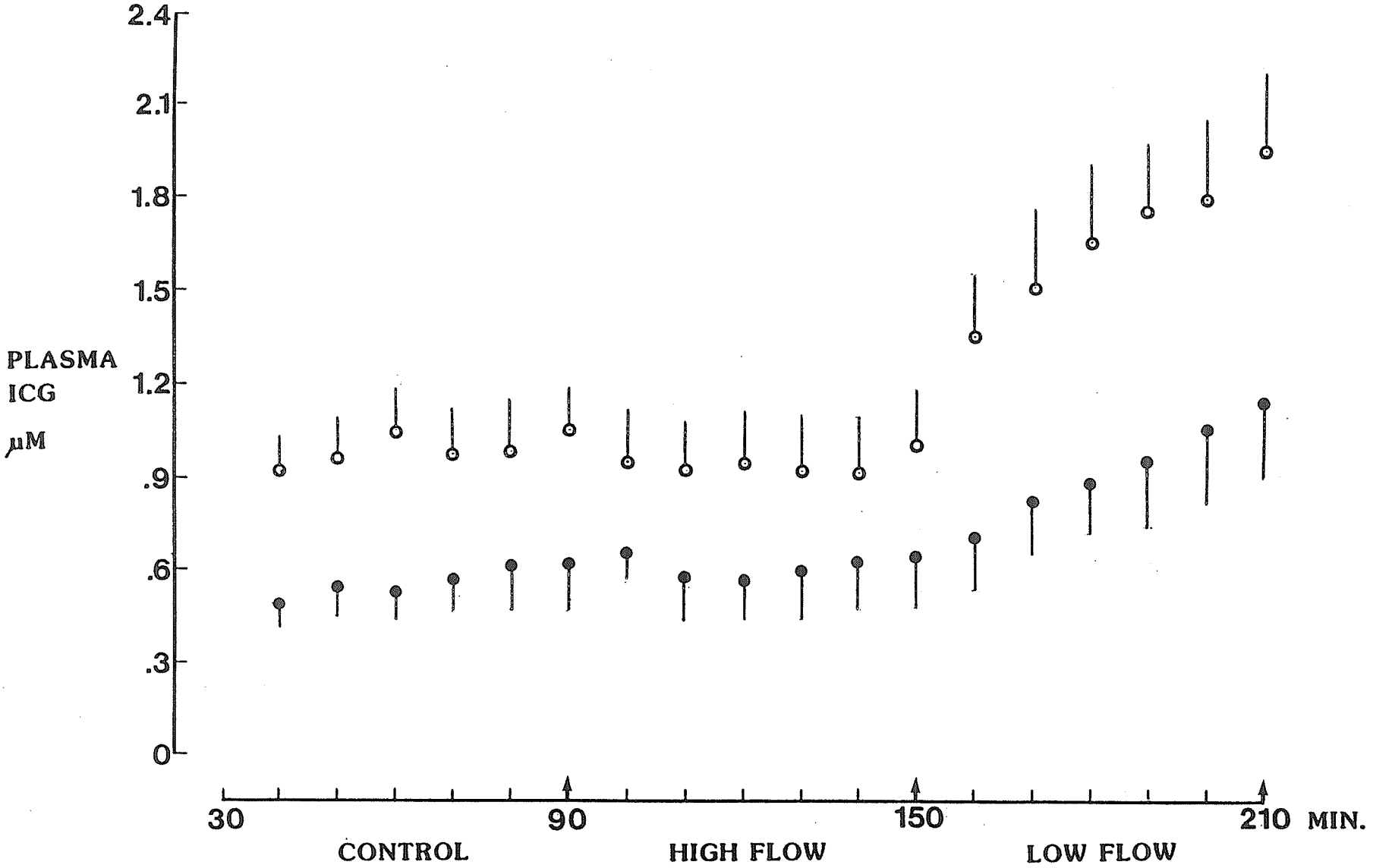

PLASMA FLOW
ml · min⁻¹ · 100 g⁻¹
140
120
100
80
60
40
20
0
E
M
C
30
90
150
210 MIN.
CONTROL
HIGH FLOW
LOW FLOW

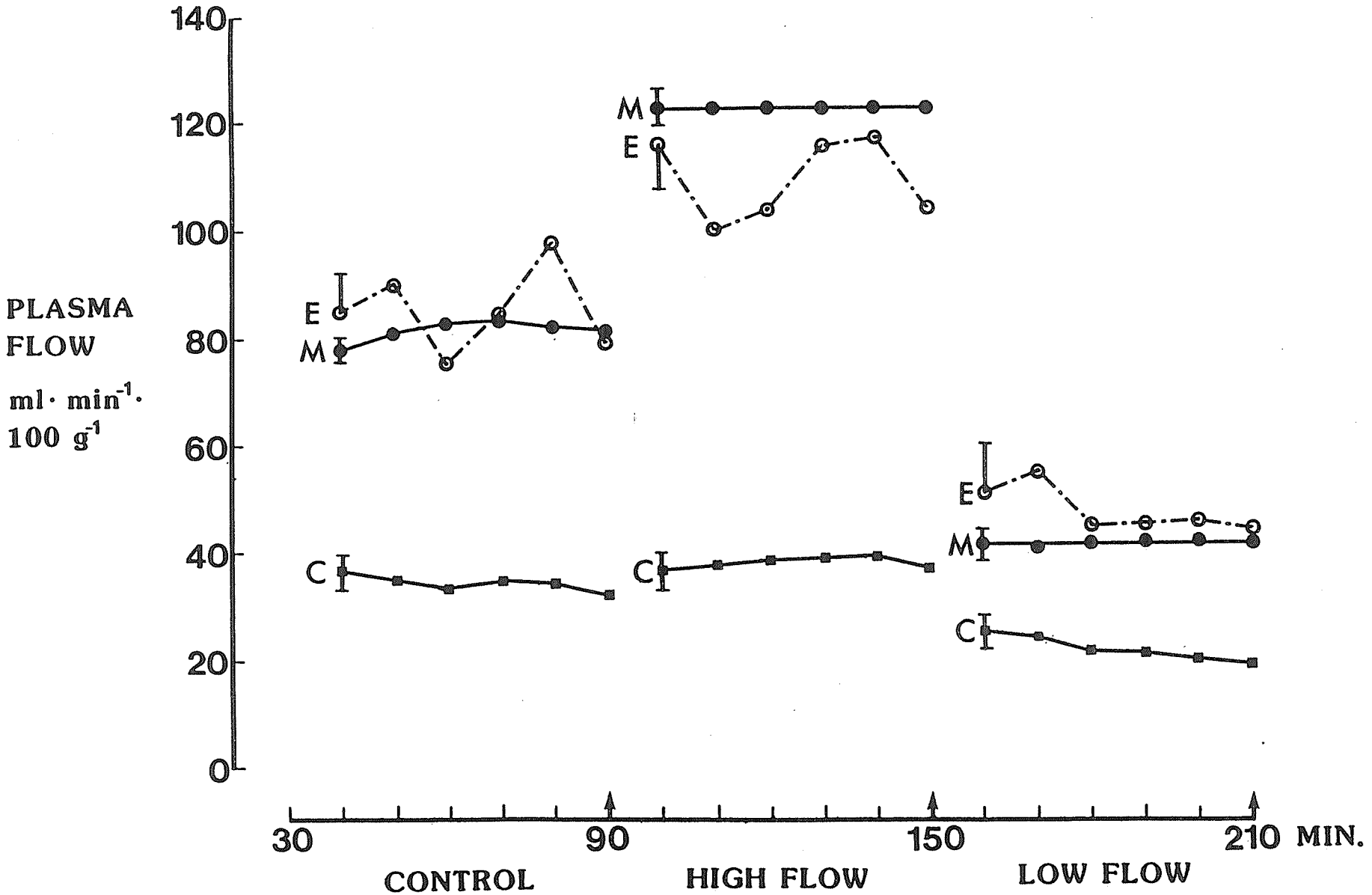

HEPATIC PLASMA FLOW
ml·min-1·100g-1
0
20
40
60
80
100
120
140
160
DIRECT
Vd.β

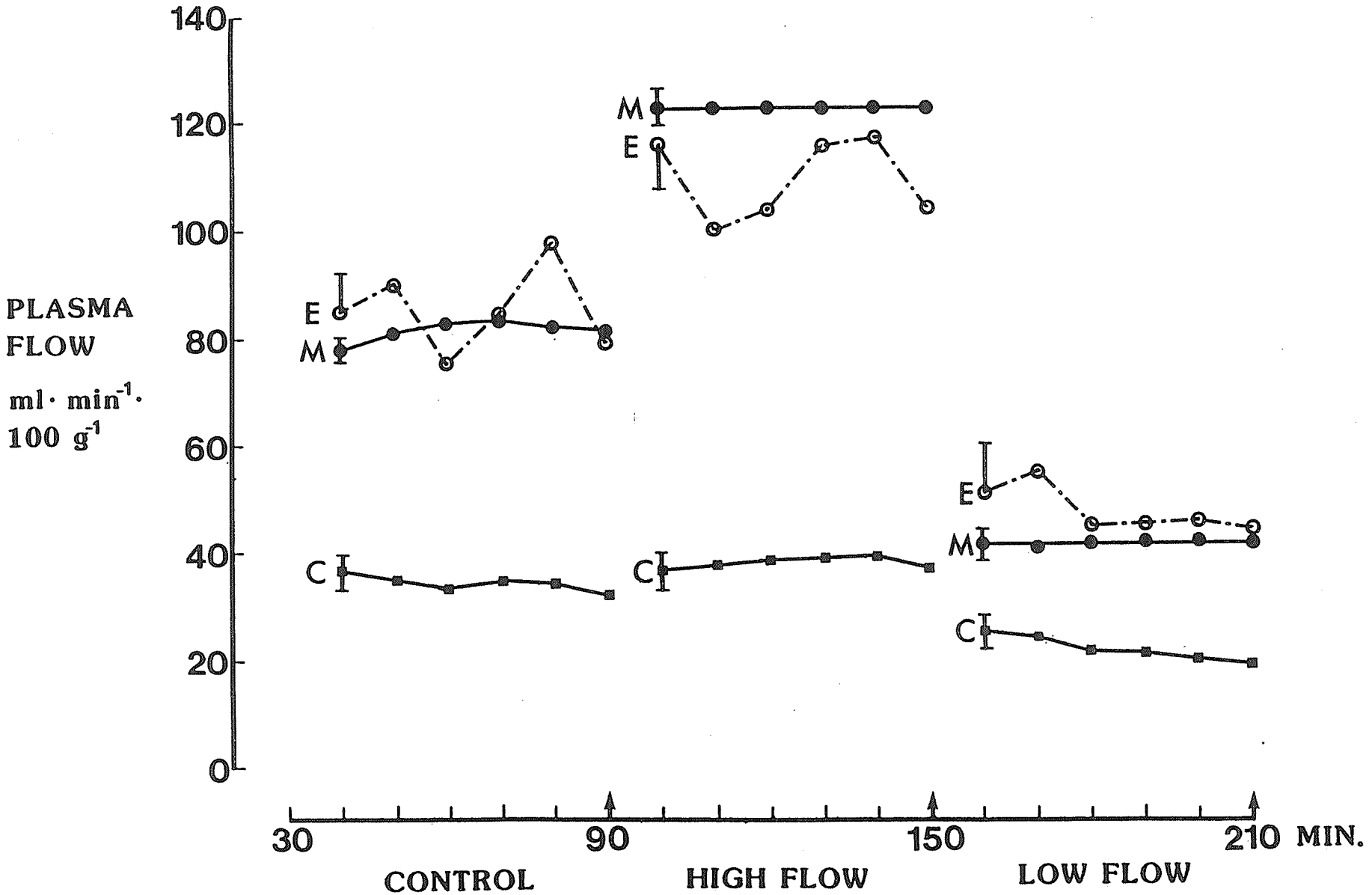

160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹


200
180
160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
EHPF ml·min⁻¹·100g⁻¹
MEAS. HPF ml·min⁻¹·100g⁻¹


PLASMA
ICG
µM
1.5
1.2
.9
.6
.3
0
60
120
180
240 MIN.
3.22
6.44
ICG nmol·min⁻¹·kg⁻¹


2.4
2.1
1.8
1.5
1.2
.9
.6
.3
0
PLASMA
ICG
µM
30
90
150
210
MIN.
CONTROL
HIGH FLOW
LOW FLOW


PLASMA FLOW
ml · min⁻¹ · 100 g⁻¹
140
120
100
80
60
40
20
0
E
M
C
30
90
150
210 MIN.
CONTROL
HIGH FLOW
LOW FLOW


PLASMA FLOW
ml · min⁻¹ · 100 g⁻¹
140
120
100
80
60
40
20
0
E
M
C
30
90
150
210 MIN.
CONTROL
HIGH FLOW
LOW FLOW

stabilization period was not recorded. Clearance showed some tendency to decrease during the control flow period but less than in the previous study. When flow was increased by 50%, clearance increased by 12%, but when flow was reduced by 50%, clearance decreased by 38% compared to the control value. Mean values of systemic clearance were 34 $\pm$ 1, 38 $\pm$ 2 and 21 $\pm$ 1 ml/min/100g liver at control, high and low flows respectively. These values were all different ($P<0.01$) by blocked ANOVA and Duncan's test. Mean hepatic clearances, calculated as uptake divided by arterial plasma concentration, were not different from the above calculated clearances. The measured plasma flows were 81 $\pm$ 2, 122 $\pm$ 3 and 41 $\pm$ 1 ml/min/100g liver for the control, high and low flow periods. As in the previous series, the individual values of estimated hepatic plasma flow (calculated as systemic clearance / extraction) at different times were highly variable (fig. 37). Mean estimated flows were 85 $\pm$ 5, 109 $\pm$ 6 and 47 $\pm$ 3 ml/min/100g liver for the control, high and low flow periods respectively. Measured and estimated mean plasma flows were not different during the control and low flow periods but estimated flow was less than measured flow during the high flow period ($p<0.05$).

To examine the reliability of the estimates of hepatic plasma flow relative to the measured flow, they were

expressed as ratios. The results, shown in figs. 38 and 39 for the fourth and fifth series, illustrate the variability in estimated hepatic plasma flow between animals at the different hepatic plasma flow rates.

E. GALACTOSE INTRAVENOUS INFUSIONS

Hematocrit at the start of the galactose infusion experiments was 36 ± 1% and 32 ± 1% at the end. Liver weight was 24.4 ± 1.1 g/kg body weight.

In 5 cats, hepatic blood flow was maintained at the initial control level (series six). Mean flow was 119 ± 7 at the start and 117 ± 7 ml/min/100g liver at the end and these values were not significantly different. Galactose was infused at four rates for 60 min at each rate. Mean arterial pressures were 131 ± 9, 152 ± 6, 147 ± 9 and 139 ± 8 mm Hg for each of the infusion periods respectively. For each infusion rate, after a constant blood concentration had been reached over 20 min, total splanchnic galactose uptake was calculated as the total hepatic flow times the arterio-hepatic venous difference for galactose. Hepatic uptake was calculated from the portal-hepatic venous difference times two-thirds of the flow plus arterio-hepatic venous difference times one-third of the

Fig. 38. Estimated hepatic plasma flows relative to measured plasma flows for each time period during the two infusion rates (series four). Individual values for the 4 cats are shown by the 4 symbols.

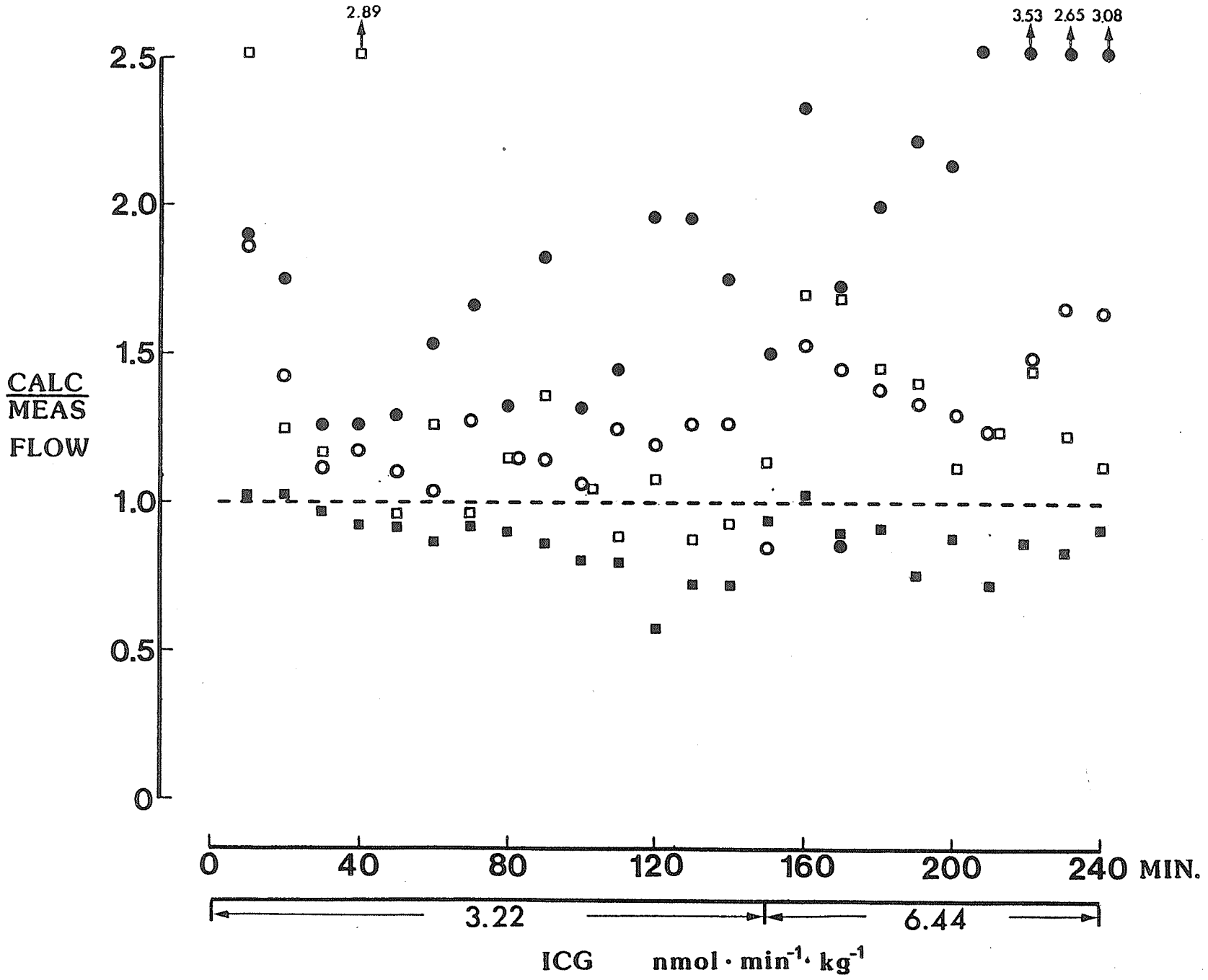
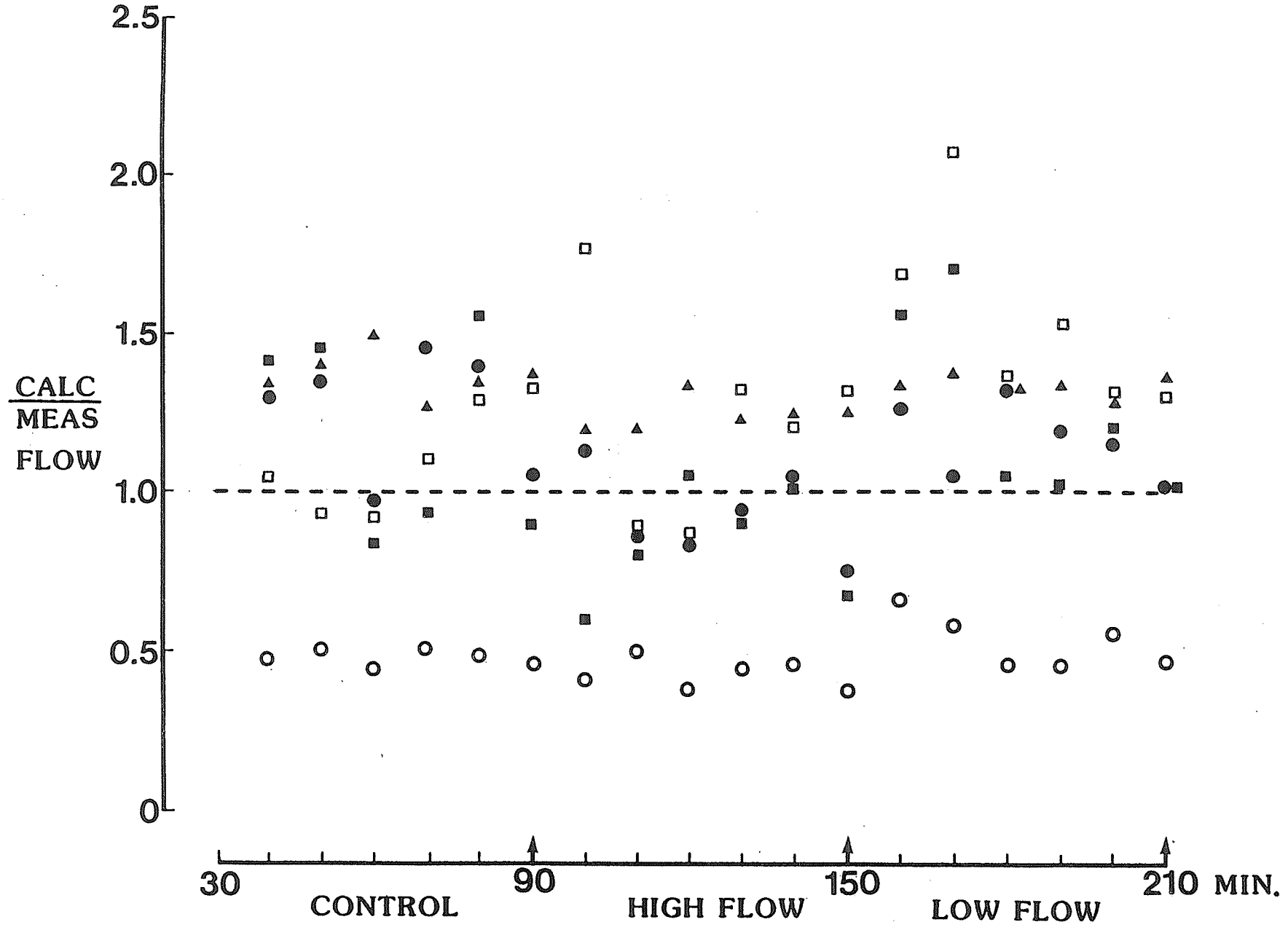

2.89
3.53
2.65
3.08
2.5
2.0
1.5
1.0
0.5
0
CALC
MEAS
FLOW
0
40
80
120
160
200
240 MIN.
3.22
6.44
ICG nmol · min-1 · kg-1

Fig. 39. Estimated hepatic plasma flows as a proportion of measured hepatic plasma flows for each time period during the 3 flow levels (series five). Individual values for the 5 cats are shown by the 5 symbols.


2.5
2.0
1.5
1.0
0.5
0
CALC
MEAS
FLOW
30
90
150
210 MIN.
CONTROL
HIGH FLOW
LOW FLOW

flow. This assumes hepatic arterial flow is one third of the total (see Introduction and Discussion). Splanchnic and hepatic uptakes, expressed as percent dose, are tabulated in Table 4. Expressed as percent dose, total splanchnic uptakes were not significantly different at the different infusion rates and averaged 77 $\pm$ 2% (range 65-90% in different cats). Similarly hepatic uptakes expressed as percent dose were not significantly different at the different infusion rates and averaged 61 $\pm$ 2% (range 45-73% in different cats). It is clear that a substantial amount of extrahepatic (39%) and extrasplanchnic uptake (23%) occurred. This was not dose dependent up to 20 umol/kg body weight/min but varied substantially among the different cats.

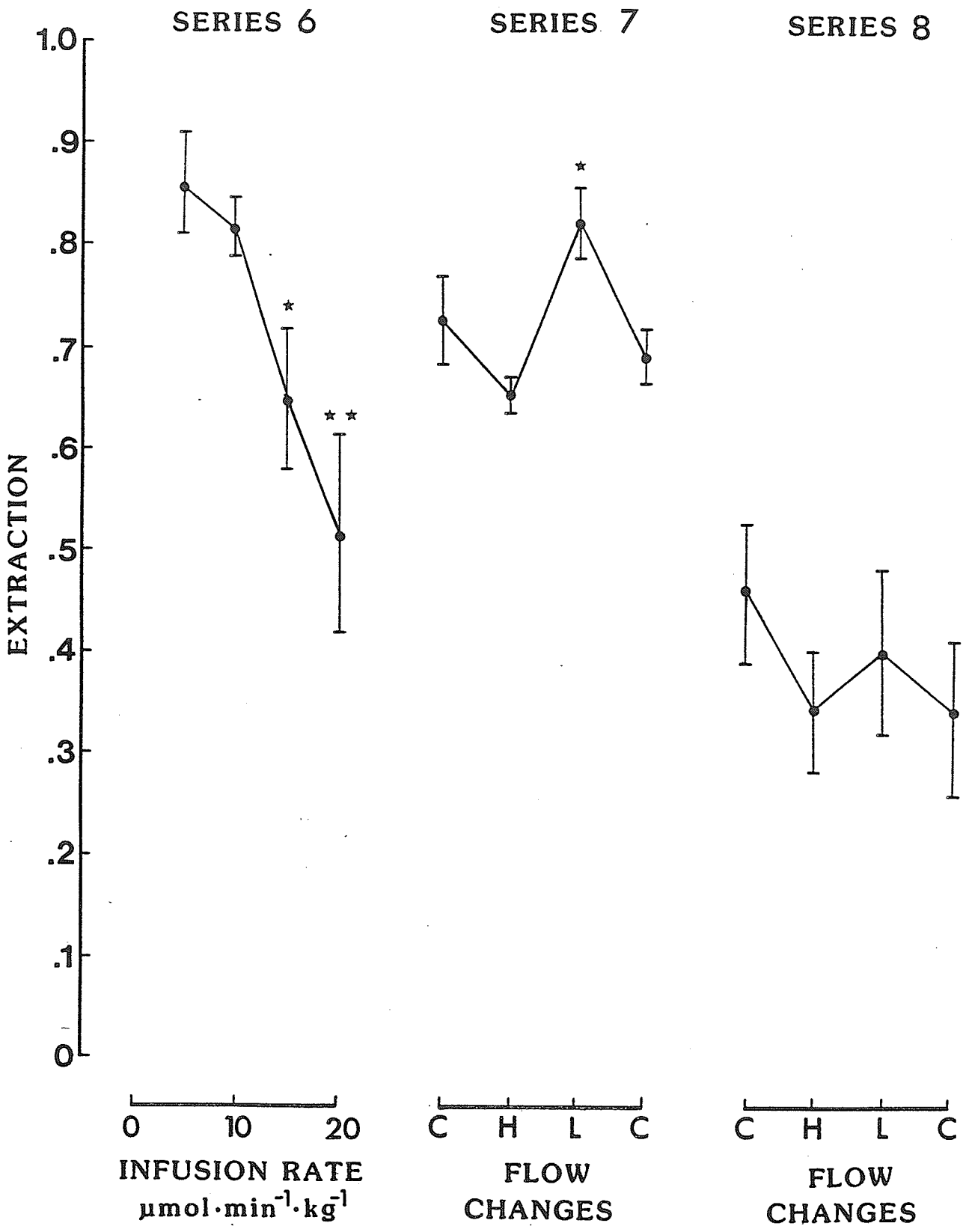
Extraction of galactose was calculated for each infusion rate after a constant blood concentration had been reached for 20 min. The results are shown in fig. 40. Extraction decreased as the infusion rate increased but the extent of the decrease was quite variable among the different animals. Blocked ANOVA showed that the decline with dose was highly significant ($P<0.01$). Even at the lowest infusion rate, which gave blood levels which could be reliably measured, extraction was less than 100%.

Systemic clearance and estimated hepatic blood flow

Table 4. Splanchnic and hepatic galactose uptake during the three experimental series.

	Uptake Expressed as Percent Dose	
	Splanchnic	Hepatic
Series 6 (Control flow) (n=6)		
5 (µmol/kg body wieght/min)	77 ± 3	62 ±3
10 (µmol/kg body weight/min)	76 ± 4	64 ± 3
15 (µmol/kg body weight/min)	78 ± 2	60 ± 3
20 (µmol/kg body weight/min)	75 ± 3	58 ± 5
Series 7 (10 µmol/kg body weight/min) (n=4)		
Control flow	82 ± 5	69 ± 5
High flow	86 ± 2	74 ± 4
Low flow	74 ± 6	62 ± 5
Control flow	82 ± 2	73 ± 4
Series 8 (25 µmol/kg body weight/min) (n=8)		
Control flow	72 ± 3	
High flow	75 ± 3	
Low flow	56 ± 5	
Control flow	74 ± 4	

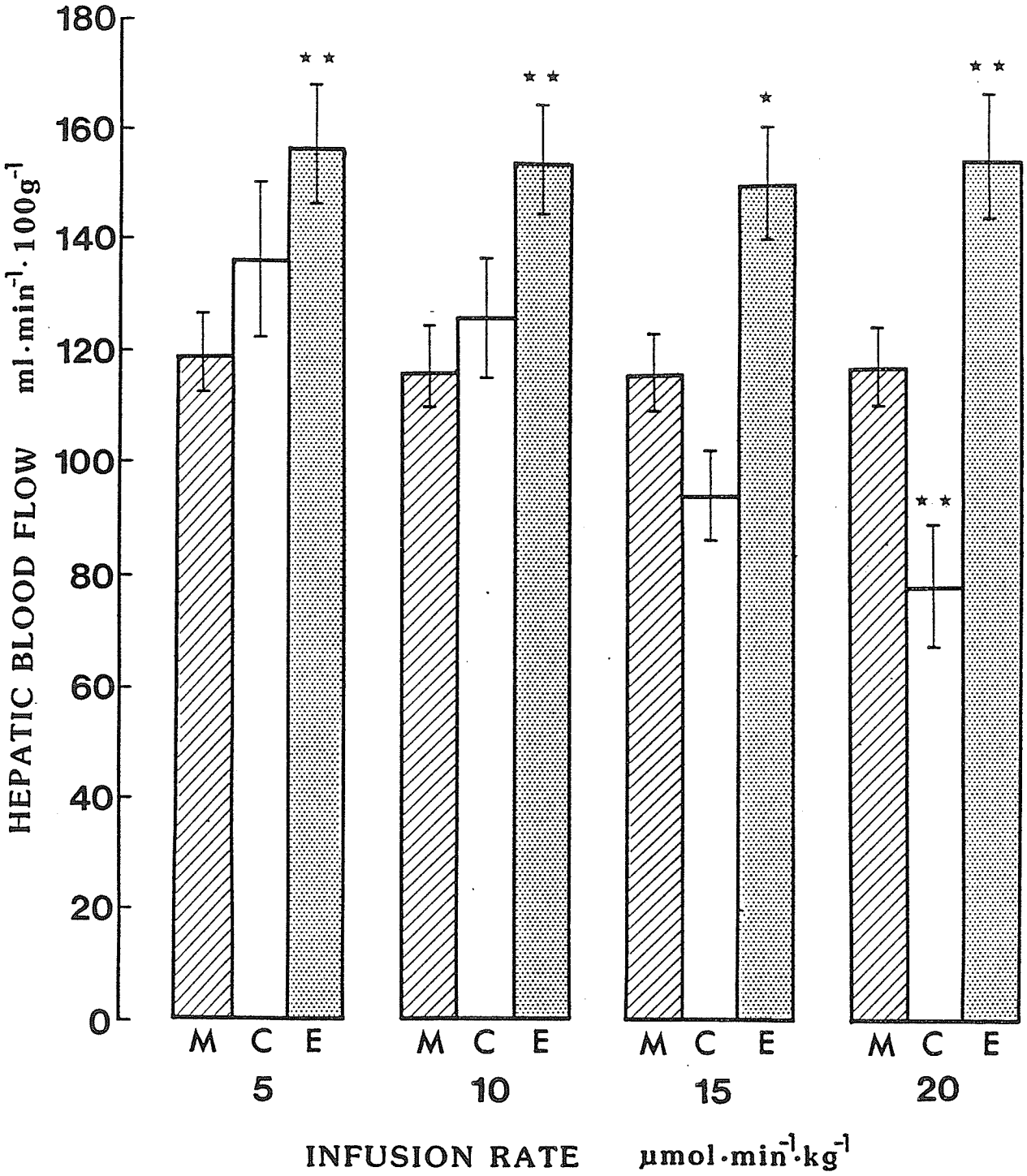
Fig. 40. Splanchnic extractions of galactose in the three series of galactose experiments. Means ± S.E.; N = 5, 4 and 8 for the three series, respectively. * P<.05; ** P<.01. For series 7 and 8, C is control flow, H is 150% control flow, L is 50% control flow.


SERIES 6
SERIES 7
SERIES 8
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
EXTRACTION
0
10
20
INFUSION RATE
µmol·min⁻¹·kg⁻¹
C
H
L
C
FLOW
CHANGES
C
H
L
C
FLOW
CHANGES

was calculated as that for the ICG infusion series and compared with the directly measured hepatic blood flows in fig. 41. It can be seen that estimated hepatic blood flow exceeded measured flow by some 30% at all infusion rates and this difference was significant ($P<0.05$). Systemic clearance decreased relative to measured flow as the infusion rate was increased, but was not significantly different except at the highest infusion rate.

In the seventh series ($N = 4$), galactose was infused at a constant rate (10 µmol/kg body weight/min) while hepatic flow was varied. The mean flows were 116 ± 8, 177 ± 13, 62 ± 4 and 117 ± 8 ml/min/100g liver during the four flow periods respectively. Mean arterial pressures were 123 ± 14, 131 ± 13, 98 ± 14 and 117 ± 13 mm Hg for the four periods respectively. After a constant blood concentration had been reached for 20 min at each flow rate, total splanchnic galactose uptake and hepatic galactose uptake were calculated as described for series 6. These values, expressed as percent dose, are also shown in Table 4. The values at the different flows were not significantly different (blocked ANOVA). Splanchnic uptake was 81 ± 2% and hepatic uptake was 69 ± 3% of the infusion rates. Extraction at the different flows is shown in fig. 40. It increased significantly during the low flow period.

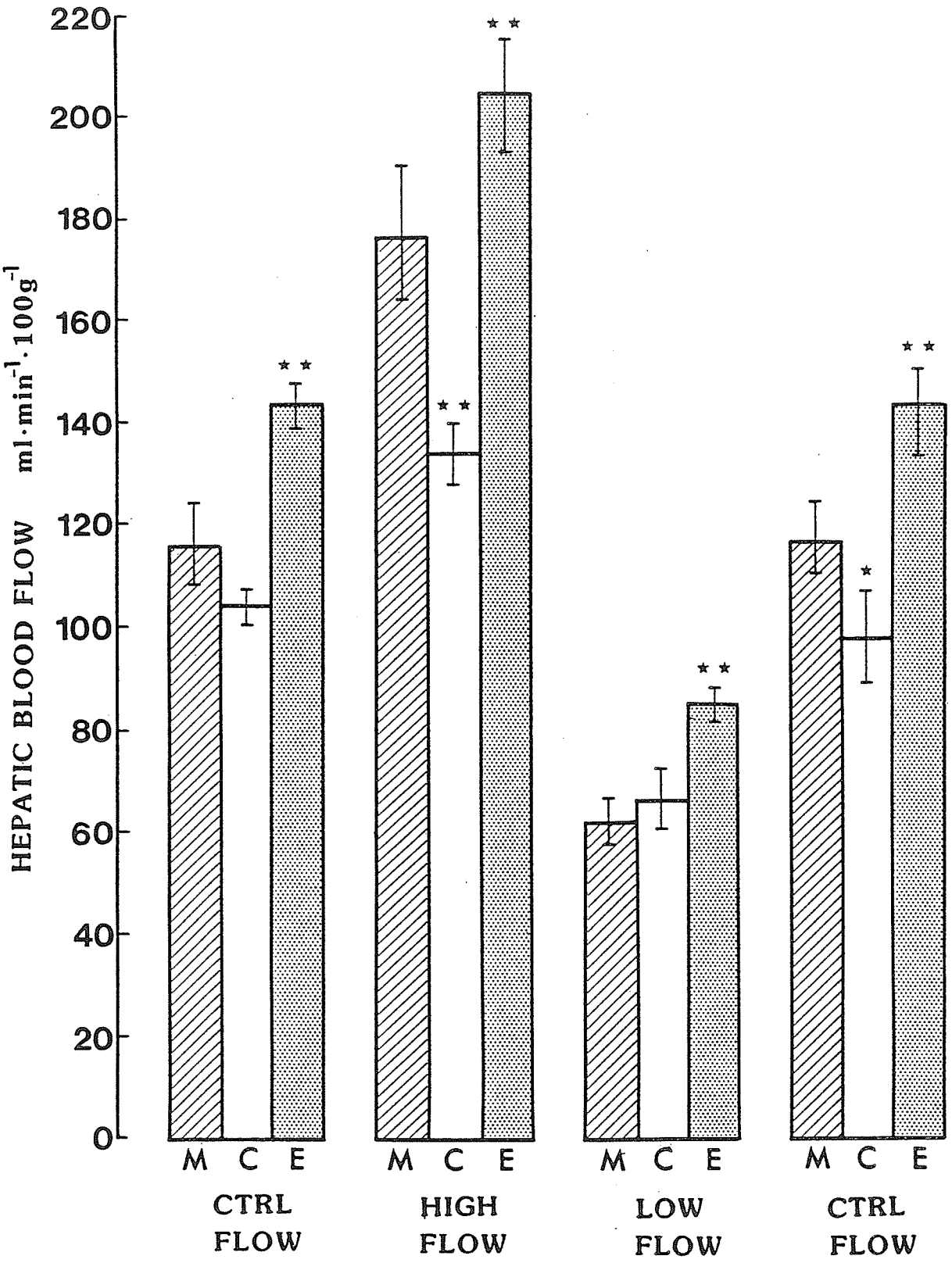
Fig. 41. Directly measured hepatic blood flow (M), systemic galactose clearance (C) and estimated hepatic blood flow (E, systemic clearance divided by extraction) at each of the 4 infusion rates in series 6. Means $\pm$ S.E. N = 5. * $P<0.05$, ** $P<0.01$ compared to the measured flow.


HEPATIC BLOOD FLOW ml·min⁻¹·100g⁻¹
180
160
140
120
100
80
60
40
20
0
M C E
5
10
15
20
INFUSION RATE µmol·min⁻¹·kg⁻¹

The measured flows, systemic clearances and estimated hepatic blood flows for this series are shown in fig. 42. Estimated hepatic flow again consistently and significantly exceeded the measured flow. Systemic clearance was close to measured flow during the first control and low flow periods but underestimated measured flow during the high flow and second control periods. Note that the S.E.s shown in the figure incorporate the variability among animals, while the statistical analysis was blocked to reduce this source of variability.

In series 8 (N = 8), galactose was infused at a higher rate (25 µmol/kg body weight/min) throughout the experiments while the flow was varied as in Series 7. Directly measured flows were 129 ± 7, 191 ± 13, 65 ± 3 and 128 ± 7 ml/min/100g liver for each flow period respectively. Mean arterial pressures were 112 ± 8, 134 ± 6, 88 ± 9 and 118 ± 7 mm Hg for the four periods respectively. Portal galactose levels were not measured in this series and only total splanchnic uptake could be calculated. Mean splanchnic uptake was 72-75% of the infusion rate during the control, high flow and final control periods, but mean uptake dropped significantly to 56% of the infusion rate during the low flow period (see Table 4.). Extraction was lower and variable in this series due to the higher infusion rate. It did not change

Fig. 42. Directly measured hepatic blood flow (M), systemic galactose clearance (C) and estimated hepatic blood flow (E), systemic clearance divided by extraction) during control flow (CTRL), high flow (150% control) and low flow (50% control) periods in series 7. Mean $\pm$ S.E. N = 4. * $p<0.05$, ** $p<0.01$


HEPATIC BLOOD FLOW ml·min⁻¹·100g⁻¹
220
200
180
160
140
120
100
80
60
40
20
0
M C E
CTRL FLOW
HIGH FLOW
LOW FLOW
CTRL FLOW

significantly at the different flow levels (fig. 40).

Measured flow, systemic clearances and estimated hepatic blood flows in series 8 are shown in fig 43. Estimated hepatic flows markedly exceeded the measured flows during all flow periods while systemic clearances were significantly less than measured flows during all the periods except the low flow. Estimated flows and systemic clearances were much more variable from animal to animal than were the measured flows. The variability in estimated flow for all the galactose experimental series is shown in Fig. 44.

In 3 hepatectomized cats, arterial blood galactose levels were measured for 94 min after a single bolus dose (1250 μmol/kg body weight). Arterial pressure was 85 mm Hg in all experiments. The mean hematocrit was 32 $\pm$ 1%. The arterial galactose concentration-time relationship is illustrated in fig. 45. Galactose levels had declined to about 10% of the initial level after 90 min. These data substantiate that a significant amount of galactose is removed by organs other than the liver.

Fig. 43. Directly measured hepatic blood flow (M), systemic galactose clearance (C) and estimated hepatic blood flow (E, systemic clearance divided by extraction) during control flow (CTRL), high flow (150% control) and low flow (50% control) periods in series 8. Means $\pm$ S.E. N = 8, * $p<0.05$, ** $p<0.01$.

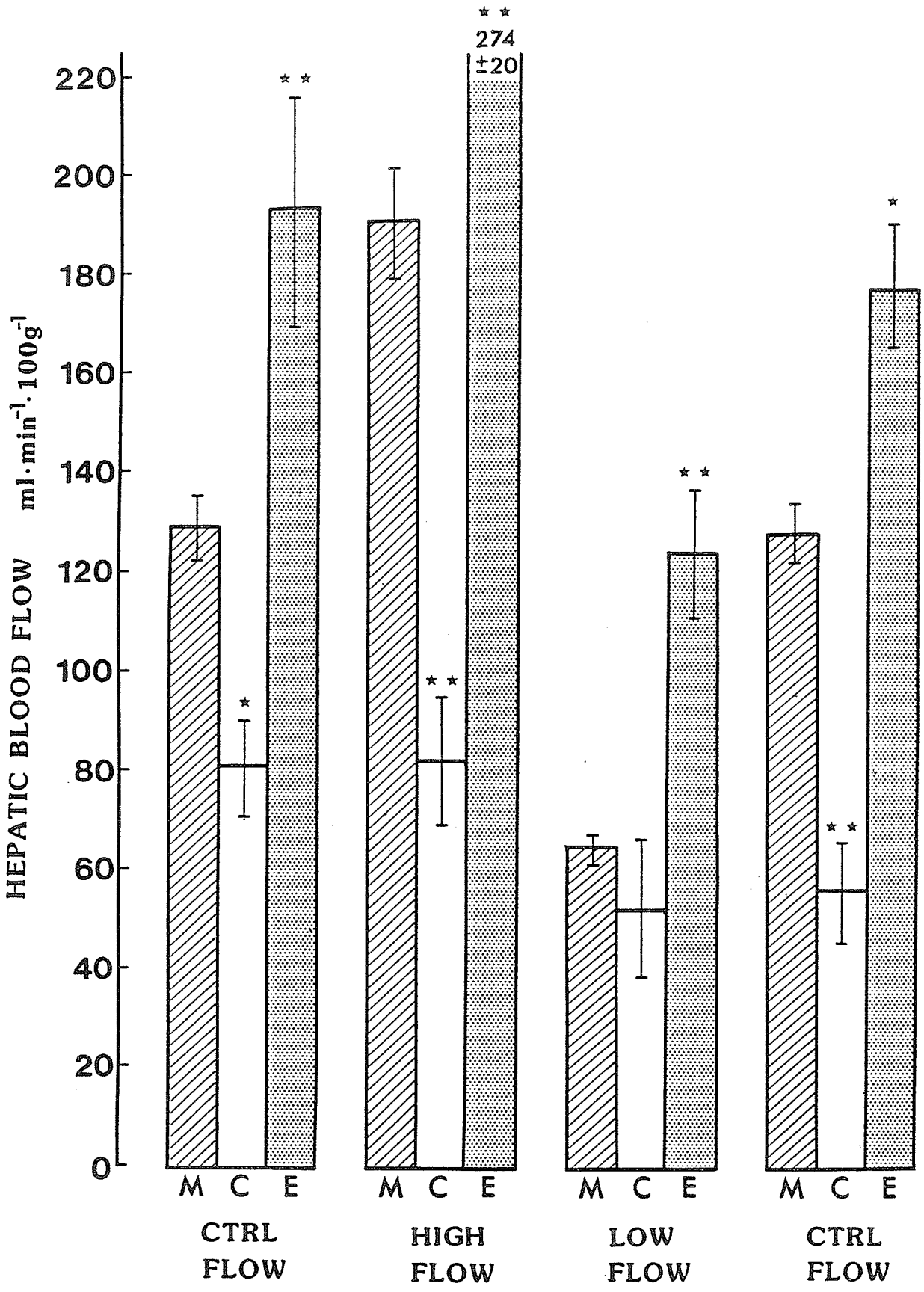

HEPATIC BLOOD FLOW ml·min⁻¹·100g⁻¹
0
20
40
60
80
100
120
140
160
180
200
220
** 274 ±20
*
**
**
**
*
M C E
M C E
M C E
M C E
CTRL FLOW
HIGH FLOW
LOW FLOW
CTRL FLOW

Fig. 44. Estimated hepatic blood flow plotted against directly measured flows during the three galactose experimental series. Symbols: Series 1, (■) 5, (○) 10, (△) 15, (□) 20 umol/kg body weight/min; Series 2 (▲); Series 3 (●).

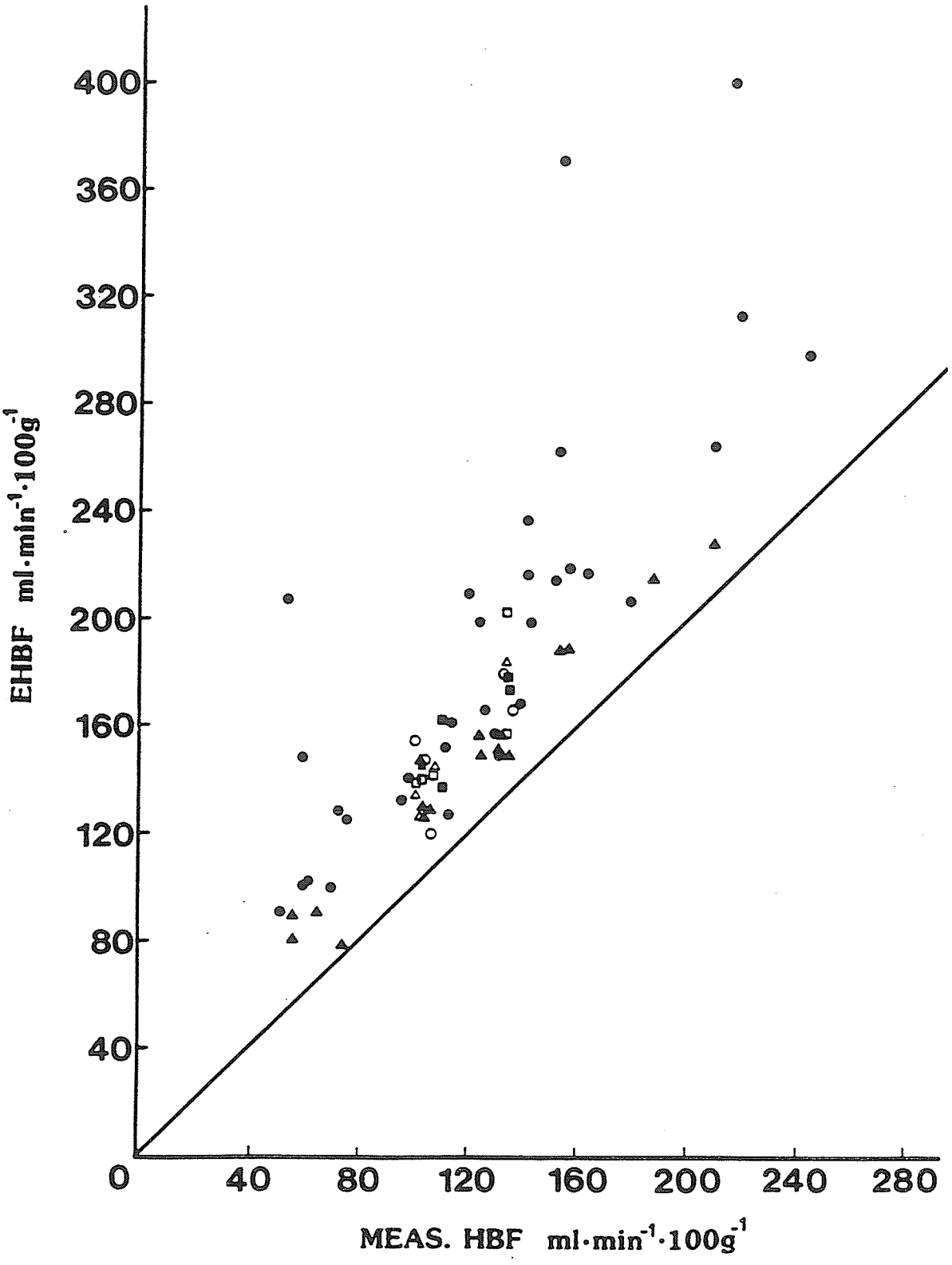
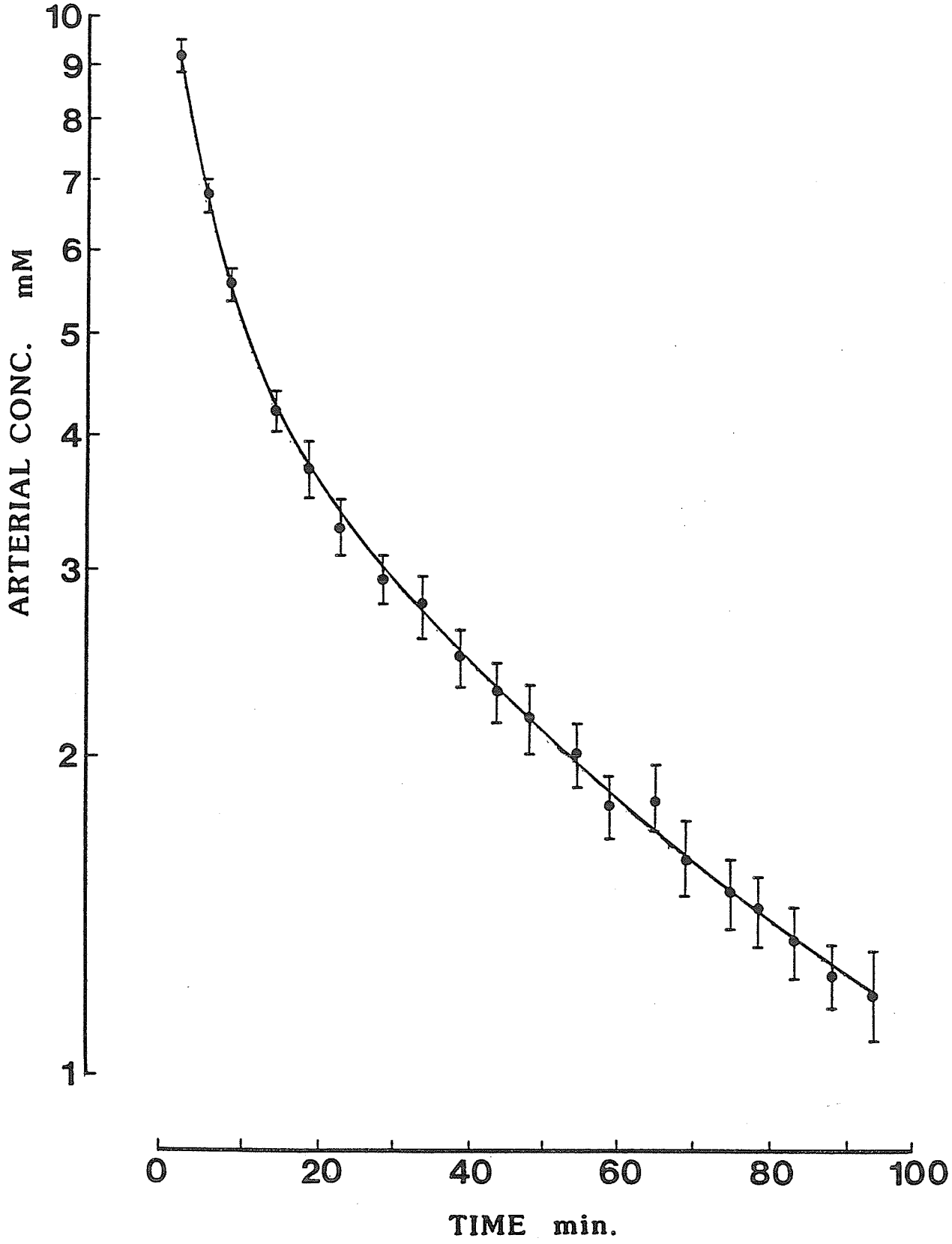

400
360
320
280
240
200
160
120
80
40
0
40
80
120
160
200
240
280
EHBF ml·min⁻¹·100g⁻¹
MEAS. HBF ml·min⁻¹·100g⁻¹

Fig. 45. Arterial galactose concentrations over 90 min after a bolus dose (1250 umol/kg body weight) in 3 hepatectomized cats. Means $\pm$ S.E.


ARTERIAL CONC. mM
10
9
8
7
6
5
4
3
2
1
0
20
40
60
80
100
TIME min.

DISCUSSION

A. ANALYTICAL METHODS

Acetonitrile has previously been used for the extraction of ICG from plasma and bile samples (156). In this extract ICG has been reported to be stable for approximately 30 min. The addition of 4% methanol to acetonitrile was found to increase stability for up to 315 min (Fig. 6) and allowed samples to be injected by an autoinjector.

The method described for HPLC analysis demonstrated lower inter-assay coefficients of variation than the SPEC method (Table 1). Greatest variability was found in the the 51.6 nmol/ml and 1.3 nmol/ml plasma samples using the SPEC method of analysis. It is known that ICG forms aggregates at high concentrations (105, 178). The formation of aggregates could cause the underestimation of ICG concentrations using the SPEC method of analysis and thus give a wide variation for the apparent concentrations (Table 1). At lower concentrations (i.e. 1.3 nmol/ml plasma) the higher coefficient of variation determined by the SPEC method could probably be due to lack of instrument sensitivity. Below this concentration (i.e. < 1.3 nmol/ml plasma) the coefficient of variation will be low and relatively constant since it is comprised of background absorbance which would be expected to be relatively constant.

A greater underestimation was found at higher biliary ICG concentrations when analyzed by the SPEC method. Although all samples were analyzed in the linear range for the SPEC analysis, a true linear relationship between optical density and concentration may not exist (105). This may be due to aggregate formation. The slight deviation from linearity together with the dilution factor and analytical error could account for the lower values obtained.

HPLC analysis of plasma, liver and bile samples demonstrated the presence of two compounds. One was identified as ICG and the other was not identified. Similar results have been reported by others (37, 156, 193). These studies suggest that the SPEC method yields erroneously low clearance rates and that the HPLC method should be used to determine plasma ICG concentrations such that reliable clearance rates may be obtained. This study demonstrated that both the HPLC and SPEC methods yield reliable plasma ICG concentrations if they are analyzed within the linear range. This is further supported by the complete recovery of ICG from liver and bile samples. Sevensson, et al. (181), also reported that the HPLC and SPEC methods yield similar estimates of ICG concentration in plasma of humans at clinically relevant doses (0.6 μmol/kg body weight).

Using chromatographic procedures, Barbier and DeWeerdt (5) had reported the existence of a degradation product when ICG was reconstituted in the manufacturer's solvent. Utilizing infrared spectrophotometry they proposed a chemical structure for the degradation product. According to their proposed structure, the degradation product's peak light absorbing capacity should be well below that of ICG (since there are less conjugated double bonds in the tentative product, peak absorption will be shifted toward the ultraviolet region). When ICG was reconstituted in either the manufacturer's solvent or plasma the same retention time for the unknown substance was found. The absorption maximum was not shifted toward the ultraviolet region but remained at 783 nm using HPLC analysis. This suggests that the degradation product reported by Barbier and DeWeerdt (5) may not be the same substance found in this study.

Following the administration of each bolus dose, slightly higher apparent ICG concentrations for each plasma sample were determined by the SPEC method. The higher concentrations obtained by the SPEC method were consistent with the increase in peak height of the unknown substance detected by HPLC analysis after successive ICG doses. It is not known whether this substance was administered to the

animals or whether it was an in vivo degradation product or metabolite since it cannot be quantitated in this study. If it was administered, the substance is extracted by the liver and is eliminated via biliary excretion.

B. ICG BOLUS DOSE ADMINISTRATION

ICG is thought to follow dose-dependent kinetics (81, 95, 190). Lower apparent elimination rates are expected as larger doses are administered. In the first series of experiments, the apparent elimination rate constant progressively decreased with successive 1.3 µmol/kg body weight bolus doses. Extraction of ICG paralleled the declining apparent elimination rate constant. Since extraction is calculated by the arterio-venous concentration difference it is technically indicative of splanchnic extraction and not hepatic extraction. The decrease in extraction may, therefore, be due to saturation of the extrahepatic sites. In some experiments, portal venous blood was sampled and analyzed for ICG concentration. These concentrations were similar to those of the arterial blood. Thus, the calculated extractions may be assumed to be hepatic and the decrease was probably not due to saturation of extrahepatic sites. The decrease in extraction was found to correlate with the amount

remaining to be excreted by the liver (accumulation of ICG by the liver). The mechanism by which the amount present in the liver is responsible for the decrease in extraction is not known. The decrease in extraction is probably not an artifact related to the deterioration of the preparation. Blood gases, pH and blood glucose were shown to be stable for several hours using this preparation (107, 113, 114). Stability of the preparation has also been investigated through galactose uptake studies which demonstrated that the V_{max} and K_m for galactose were similar after several hours. Further experiments utilizing radio-labelled ICG may elucidate the pharmacokinetics of this organic anion.

Estimations of hepatic plasma flow from ICG systemic clearances were unsatisfactory. Clearances alone gave values much below the measured flows since extraction was quite low in these cats. Higher extractions might be expected with smaller doses, but even the HPLC method is not sufficiently sensitive to measure the resulting plasma concentrations over an adequate time period. Extractions close to 100% have never been reported in either humans or animals. This series of experiments demonstrated that systemic clearance of ICG, following bolus doses, cannot be used as an estimate of hepatic plasma flow.

Estimation of hepatic plasma flow by correction of systemic clearances for hepatic extraction gave values which overestimated the measured hepatic plasma flows. Conversely back-calculation of hepatic extraction from the measured flows gave higher values than the calculated extractions. These discrepancies could arise in two ways: if systemic clearance overestimated hepatic clearance due to extrahepatic distribution and uptake of ICG, or if hepatic extraction underestimated the true extraction due to transit time problems. Both of these possibilities could play a role in these experiments.

Data from the first series of experiments indicated that hepatic clearance was much less than systemic clearance after the first two doses. The estimated flow greatly exceeded the measured flow rate during this period. Upon successive ICG administrations, the estimated flow approached the true hepatic flow rate (although still significantly greater than the measured). This observation suggests that uptake into tissues other than the liver is greatest for the initial dose. With successive doses, uptake into extrahepatic sites may decrease, possibly due to saturation.

Since at 6h (first series), 97% of the administered ICG was recovered in liver tissue and cumulated bile and

87% was recovered after 4h (second series), any such extrahepatic redistribution must be temporary. The kidney was found to be a major organ responsible for extrahepatic uptake of ICG. The possibility that the kidney is acting only as a temporary reservoir seems likely since ICG was found in the urine of only 1 of 10 cats. Furthermore, the possibility that some ICG distributes into extrahepatic tissues while the plasma concentration is high, returning to the plasma as the plasma concentration falls, and is ultimately taken up by the liver is also likely. Such a redistribution could account for the slight curvature of the plasma concentration time curves (Fig. 10 and 11).

When the extrahepatic uptake rate constant, determined in the hepatectomized series, was subtracted from the elimination rate constants of the first series, the estimated hepatic plasma flow (corrected for extraction) was similar to the measured flow. If the rate constant was assumed to be independent of flow changes and subtracted from those of the second series, then the estimated hepatic plasma flow closely approximated the measured plasma flow. The conventional method of calculating hepatic blood flow uses the initial linear phase of the concentration-time relationship. This portion of the curve represents distribution as well as elimination. If a biexponential equation was assumed to describe the data, then beta (the

terminal phase of the concentration-time relationship) represents hepatic elimination as well as redistribution. When hepatic blood flow was calculated using beta, the estimated flow was similar to the measured flow. The results, however, were again highly variable. Thus, temporary extrahepatic distribution of ICG seems likely to explain at least part of the overestimation of the hepatic plasma flows. The variability in estimating flow among animals may be due to transit time problems for obtaining the hepatic venous blood samples and the subsequent calculation of extraction.

Accurate measurement of extraction requires measurement of ICG in the same blood before and after it has passed through the liver. Since arterial ICG concentrations are falling rapidly, the time interval between the arterial sample and the hepatic venous sample is critical and must equal the transit time. We used an estimated average transit time of 15 s. Calculation of the true transit time is complex. Blood entering the liver consists of hepatic arterial blood (about 33%) which would be expected to contain an ICG concentration similar to femoral arterial blood, and portal blood (about 67%) which would be expected to contain a higher ICG concentration since it left the arterial system one intestinal transit time earlier. Thus, the concentration of blood entering the

sinusoids would be expected to have a higher ICG concentration than a simultaneous arterial sample. Since both intestinal and hepatic blood volumes can vary considerably in different animals (63), the determination of true extraction is extremely complex. When arterial blood concentrations are changing with a rate constant of 0.10-0.15 min^{-1} (Fig. 10), these uncertainties can introduce errors of up to 20% in the determination of extraction from arterial and hepatic venous ICG concentration measurements (see Table 5). Thus, transit time may introduce significant errors in the estimation of hepatic plasma flow.

These experiments provide further evidence that the kinetics of ICG are complex. A model to characterise the kinetics of ICG has not yet been defined. The uptake rate from plasma into the liver greatly exceeded the elimination rate from the liver into bile. At 12 min after a bolus dose more than 90% of the ICG had disappeared from plasma while less than 5% had appeared in the bile. With a half-time for biliary excretion of 2.9 hours, only a small portion of each bolus dose had been excreted from the hepatocytes at 40 min when the next bolus dose was given. Thus extensive accumulation of ICG in the liver occurred. It seems reasonable to expect that net uptake will decrease as accumulation occurs and this may explain the reduced

elimination rate constants on repeated bolus doses in series 1 and the discrepancy between the first and last boluses at control flow in series 2. However separation of this effect from a time-dependent decline in uptake is difficult. Also in the plots of hepatic uptake against plasma concentration (Figs. 19 and 20), there was no indication of decreasing uptake as accumulation occurred during the course of each bolus. Since accumulation occurs as plasma concentration falls, these curves (Figs. 19 and 20) might be expected to be concave to the abscissa. Semi-logarithmic plasma concentration-time curves should become flatter with time as occurred to a small extent (Figs. 10 and 11). However ICG has been reported to show dose-dependent (Michaelis-Menten) kinetics (81, 95, 190). If this were so, the uptake curves (Figs. 19 and 20) would be expected to be convex to the abscissa, and semi-logarithmic plasma concentration-time curves should become steeper with time. It seems most likely that the apparent linearity of both sets of curves (Figs 10, 11, 19 and 20) represents a balance between opposite curvatures due to accumulation and Michaelis-Menten kinetics, and that ICG does not follow the first-order one compartment open model discussed in the Introduction. This would explain the major difficulty in the literature (81, 95, 190) that although initial uptake rates of ICG are dose dependent, the uptake does not increase as plasma concentration falls.

Thus these studies do not follow the predictions of the Michaelis-Menten type of kinetics even though they were interpreted as if they did. If such an effect of accumulation on net hepatic uptake is present, we have no data on whether it involves decreasing influx or increasing efflux of ICG. We have been unable to determine whether ICG once accumulated in the liver can diffuse back into the plasma. However after homogenization, the ICG could not be extracted from liver solids unless acetonitrile/methanol was added and this might suggest that passive efflux by diffusion will not occur. Thus several questions must be elucidated before a satisfactory quantitative model of ICG kinetics can be accepted.

C. ICG INFUSIONS

Since ICG follows dose-dependent kinetics (81, 95, 190), higher extractions are expected with lower plasma concentrations. In this study, the initial infusion rate was the lowest rate which gave hepatic venous plasma concentrations that could be analyzed accurately by the HPLC method. Even at this minimal infusion rate, steady state plasma concentrations could not be achieved. If the infusion had been stopped after 70 min, however, it might have appeared that steady state had been reached. This is

Table 5. Variables which may lead to potential errors in measurement of extraction when arterial concentration is changing rapidly.

1) Intestinal transit time - 10-25 seconds. Portal concentration > Arterial concentration (67% of hepatic blood is portal, 33% arterial).
2) Liver transit time - 7-34 seconds.
3) Sampling time - 5-10 seconds.
4) Analytical error - 5%

evident during the altered flow experiments where blood was sampled for only 60 min (Fig. 32). As the infusion rate was doubled, both the arterial and hepatic venous concentrations increased more rapidly until the infusion was stopped at 240 min.

Arterial and venous plasma concentrations decreased slightly at the high flow rate. When flow was decreased to 50% control, both arterial and hepatic venous ICG concentrations increased. The slight decrease in plasma concentrations at the high flow rate was probably due to the increased systemic clearance. Otherwise the plasma concentrations would have been expected to increase slightly as observed in the first series of experiments. Conversely, systemic clearance was much lower during the low flow period causing a much greater increase in plasma concentrations.

Initial extraction of ICG at the lowest infusion rate (fourth series) was 0.47. This extraction was greater than that obtained after bolus dose administration. The total dose administered at the end of 240 min was slightly less than the first bolus dose of the first series. The final extraction, 0.28, was similar to that of the first bolus dose. At the high flow rate, extraction decreased by 14% and at the low flow rate, it increased by 7%. Thus, during

raised flow extraction decreased while during reduced flow it increased. Similar results were obtained during bolus dose administrations of ICG and infusions of galactose. These results were not unexpected (201).

Like extraction, clearance showed a steady decline with time. The assumption that ICG is completely extracted at low doses is commonly made when it is used both clinically and experimentally. This assumption avoids the use of hepatic venous catheterization. The calculated clearance greatly underestimated true hepatic plasma flow in the anesthetized cat. When the calculated clearance was corrected for the incomplete extraction, estimated plasma flow exceeded the measured flow rate during the initial 50 min. During this period, extrahepatic distribution may be responsible for the overestimation of hepatic plasma flow. After 50 min it may no longer distribute itself into the extrahepatic sites if distribution equilibrium had been achieved. Thus, the mean estimated hepatic plasma flow reflects the true hepatic plasma flow only after this time period. As the infusion rate was doubled, estimated hepatic plasma flow again exceeded the true flow, probably due to distribution into the extrahepatic sites.

Estimated plasma flow was highly variable at all doses and all flow rates. Variability in the means ranged from

0.97 to 1.87 times measured flow during the first series of experiments. Variation as a function of animal studied and time after drug administration gave estimates of plasma flow which ranged from 0.59 to 3.53 times the measured flow. When the mean estimated flows were determined over 40-90 min into each infusion for the 4 cats (series 4), they were 1.24 and 1.32 times the measured flow, but in individual cats they varied from 0.85 to 2.89 times measured flow. During the second series of experiments, the range of the mean values were between 0.84 to 1.4 times measured flow. However, the values in individual cats varied widely from 0.36 to 2.0 times measured flow. Although some points clearly reflect random errors, in many cases it is clear that in an individual cat, estimated hepatic plasma flows over consecutive periods of time were more than 20% and in some animals 50% different from the measured flow.

D. GALACTOSE INFUSIONS

Splanchnic uptake of galactose was calculated from the arterio-hepatic venous concentration difference and the total hepatic flow. This reflects total removal by intestine, stomach, pancreas, spleen and liver. Hepatic uptake calculations must be regarded as approximate, since

hepatic arterial and portal flows were not separately measured and it was assumed that arterial flow was 33% of the total (63). This proportion would be expected to increase during the periods of low flow (58, 122). However, the error due to this assumption is small since arterial and portal galactose levels differed at most by some 20%. The uptake data in the three series of experiments show that extra-hepatic and extra-splanchnic removal of galactose was substantial. This extrahepatic removal continued over more than 6 hours and for more than 90 min in the hepatectomized cats. It seems clear that this represents uptake and removal and not simply distribution of galactose but the sites of this uptake have not yet been studied. Although mean extra-splanchnic uptake was relatively constant (20-30%) as a proportion of infusion rate for infusion rates of 5-20 μmol/kg body weight/min and for different flows, it varied widely (10-55%) in different animals and increased at the higher infusion rate of 25 μmol/kg body weight/min. It seems unlikely that extrahepatic uptake would become zero in all animals at an even lower infusion rate even if a convenient method of measuring the lower blood galactose concentrations were to become available. It is, therefore, clear that the assumption that hepatic uptake equals infusion rate for galactose is incorrect in anesthetized cats.

Calculation of estimated hepatic blood flow as systemic clearance divided by extraction will overestimate the true flow to the extent that the infusion rate overestimates splanchnic uptake. This is shown by the data. In all series, estimated hepatic flow was greater than measured flow (Figs. 41, 42 and 43) to the extent expected from the extrahepatic uptake. Until some method of measuring extrahepatic uptake is found that does not involve prior knowledge of hepatic blood flow, this approach to estimation of hepatic blood flow will not be reliable or accurate.

Further analysis of the data indicated that even estimation of percentage changes in flow were quite variable and unreliable. When measured hepatic blood flow was increased by 50%, the mean estimated change was 28% in series 7 and 2% in series 8 from the clearance measurements and 43% and 42% respectively from estimated hepatic flow measurements. When measured hepatic flow was decreased by 50%, the mean estimated change was 37% in series 7 and 35% in series 8 from clearance measurements and 41% and 35% respectively from estimated hepatic flow measurements.

The use of systemic clearance as a measure of hepatic blood flow involves two assumptions - that hepatic uptake equals the infusion rate, and that extraction is complete

during passage of blood through the liver. If extrahepatic uptake is present, systemic clearance overestimates flow, while if extraction is less than complete, it underestimates flow. Thus, these two errors, extrahepatic uptake and incomplete extraction, tend to cancel one another and if they are equal in magnitude, then systemic clearance will equal the measured hepatic blood flow. In the galactose series, the agreement was very good at a control flow with an infusion rate of 10 µmol/kg body weight/min (Figs 41 and 42), and agreement was also seen in dogs at control flow with an infusion rate of 5.5 µmol/kg body weight/min (76). However, since the two offsetting errors are independent, such agreement is fortuitous. The infusion rate and flows at which agreement will occur vary markedly and unpredictably in different animals. Thus, the agreement between systemic galactose clearance and hepatic blood flow under certain conditions does not prove the validity and accuracy of this approach. Clearance will overestimate hepatic blood flow as extraction increases, during lower doses and lower flows, and it will underestimate hepatic blood flow as extraction decreases, during higher doses and higher flows. This is shown by the data.

In 1963, Bradley (13) reviewed the clearance technique for estimating hepatic blood flow. He concluded that

"suitable test substances have been found and adequate evidence of reliability has been forthcoming to warrent qualified acceptance of much of the data set out." This conclusion was premature. Unfortunately no substance has been found which accurately reflects true hepatic blood flow. The elusive test substance is probably an endogenous product that is being overlooked. A practical non-invasive technique which could be used both clinically and experimentally "... has been [and still is] a highly desirable if somewhat elusive goal..." (12).

SUMMARY

This study in cats demonstrated that the addition of 4% methanol to acetonitrile greatly increased stability of ICG. The study also demonstrated that the two methods used to estimate ICG, SPEC and HPLC, gave equally good results provided that the ICG concentrations were analyzed in the linear range. The HPLC method was considerably more sensitive and required a much smaller sample volume for analysis. Overall, the HPLC method was approximately 40 times more sensitive than the SPEC method. An unknown peak was detected by HPLC as reported by others (37, 156, 193). Recovery of injected ICG was essentially complete from plasma, liver and bile at the end of 6 h (first series) and the volume of distribution of ICG corresponded to plasma volume in these cats (63). Distribution of ICG in the various lobes of the liver, as determined quantitatively by the ICG concentrations and qualitatively by consistancy of dye color between lobes, was uniform in all experiments. This confirms extensive previous conclusions that the liver is uniformly perfused in anesthetized animals (63).

The estimation of hepatic plasma flow from systemic clearance of ICG bolus doses without measurements of extraction gives values which are unacceptably low in comparison with the true values. Hepatic ICG extraction in cats is much lower than that of humans. Extraction, however, in both humans (4, 19, 24, 30, 102, 200, 202) and

cats is highly variable. When clearance is corrected for the incomplete extraction, the estimated values are closer to the true flow values but are highly variable due to transit time errors, and in cats they frequently overestimate the true flow. The overestimation may be due to temporary extrahepatic distribution.

Mean hepatic plasma flow estimated by a low dose infusion of ICG was found not to be a reliable indicator of true hepatic plasma flow, unless samples are taken after a prolonged infusion. In cats, reliable data are obtained after 50 min of infusion. When hepatic flow changes, the calculated flow tends to underestimate the change in both directions, i.e. it underestimates the increased flow and overestimates the decreased flow. Data obtained from any one experiment, however, are highly variable and may not reflect true hepatic plasma flow.

Systemic galactose clearance was also found to be unreliable in estimating hepatic blood flows due to considerable extrahepatic elimination. This is unfortunate, since such a simple method would be of enormous value. Until extrahepatic uptake and incomplete extraction have been proven not to occur in humans, it seems prudent to assume that this approach is also unreliable in clinical studies.

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