Aspectos do zoneamento e da regulação de fluxos de carbono e nitrogênio no fígado

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá, área de concentração em Biologia Celular, para obtenção do grau de Doutor.

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Maringá
2007

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

C728a

Comar, Jurandir Fernando
Aspectos do zoneamento e da regulação de fluxos de carbono e nitrogênio no fígado / Jurandir Fernando Comar. -- Maringá : [s.n.], 2007.
61 f. : figs.

Orientador : Prof. Dr. Adelar Bracht.
Tese (doutorado) - Universidade Estadual de Maringá. Programa de Pós-graduação em Ciências Biológicas, 2007.

1. Glutamina. 2. Perfusão de fígado. 3. Zoneamento metabólico. 4. Metabolismo hepático. I. Universidade Estadual de Maringá. Programa de Pós-graduação em Ciências Biológicas. Título.

CDD 21.ed. 572.4

Este é um trabalho de equipe, realizado no Laboratório de Metabolismo Hepático da Universidade Estadual de Maringá, formado pelos artigos:

1. **JURANDIR FERNANDO COMAR, FUMIE SUZUKI-KEMMELMEIER, ÉCIO ALVES NASCIMENTO** & **ADELAR BRACHT.** Flexibility of the hepatic zonation of carbon and nitrogen fluxes linked to lactate and pyruvate transformations in the presence of ammonia. ***American Journal of Physiology*** (prelo).

2. **JURANDIR FERNANDO COMAR, FUMIE SUZUKI-KEMMELMEIER, JORGETE CONSTANTIN** & **ADELAR BRACHT.** Hepatic zonation of carbon and nitrogen fluxes derived from glutamine and ammonia transformations (artigo original).

RESUMO

INTRODUÇÃO E OBJETIVOS — É consenso que a atividade metabólica está distribuída heterogeneamente ao longo dos ácinos hepáticos. Algumas vias predominam em células periportais e outras em células perivenosas. Evidências a favor desta visão foram obtidas em estudos sobre a distribuição de enzimas, em medidas de fluxo metabólico em hepatócitos periportais e perivenosos isolados e no fígado em perfusão e, mais recentemente, num estudo sobre a expressão diferencial de enzimas em hepatócitos periportais e perivenosos. Apesar deste consenso geral e do grande número de estudos já realizados, há vários aspectos ainda aguardando uma investigação mais detalhada. Há claras indicações de que o zoneamento apresenta certo grau de flexibilidade já que ele pode sofrer alterações quando as condições metabólicas são alteradas. Além disto, observações conflitantes não são incomuns, especialmente quando fluxos com regulação recíproca são analisados, como por exemplo, os fluxos de carbono e nitrogênio envolvendo aminoácidos e amônia. As discrepâncias mais frequentes são aquelas entre medidas de atividade enzimática ou de expressão gênica e as medidas dos fluxos metabólicos reais na célula intacta. Por exemplo, a produção de uréia devida à transformação de alanina no fígado perfundido de rato foi encontrada como tendo distribuição uniforme ao longo do parênquima hepático. Este é um resultado inesperado em virtude da observação de que a expressão gênica de várias enzimas-chave do ciclo da uréia predomina na região periportal. Além disto, vários investigadores encontraram que a atividade da alanina aminotransferase predomina em hepatócitos periportais. Embora tenha sido encontrada uma maior atividade neoglicogênica a partir de alanina na região periportal, observações paralelas sugerem fortemente que ela não se deve primariamente à predominância periportal da alanina aminotransferase e sim, provavelmente, à predominância periportal geral da via neoglicogênica.

Os resultados sobre o zoneamento hepático do metabolismo da alanina discutidos acima fazem surgir a questão sobre a real distribuição funcional da alanina aminotransferase e do ciclo da uréia ao longo dos ácinos hepáticos. Um modo alternativo de analisar a verdadeira distribuição funcional da alanina aminotransferase e do ciclo da uréia seria medir as produções de alanina e de uréia a partir de precursores tais como lactato ou piruvato mais amônia. Estes são, precisamente, os principais objetivos do presente trabalho no qual estes e vários parâmetros adicionais relacionados aos fluxos de carbono e nitrogênio foram medidos no fígado de rato em perfusão bivascular. Foi mostrado em trabalhos anteriores que a infusão de substratos através da artéria hepática em perfusão retrógrada permite atingir seletivamente células periportais em contraste com a mesma infusão em perfusão anterógrada, na qual todas as células poderão ser atingidas. Esta particularidade da microcirculação do fígado de rato proporciona um método adequado para investigar o zoneamento metabólico no órgão intacto. Espera-se que os resultados permitam obter um quadro mais completo sobre o zoneamento dos fluxos de carbono e nitrogênio derivados das transformações de amônia, lactato, piruvato e glutamina sob diversas condições.

MÉTODOS — Ratos machos Wistar, pesando entre 180 e 220 g, alimentados *ad libitum* com dieta laboratorial padrão foram usados. A ração foi retirada 24 horas antes da realização dos experimentos. O fígado foi perfundido bivascularmente no modo não-recirculante. O líquido de perfusão foi o tampão Krebs/Henseleit-bicarbonato (pH 7,4), saturado com uma mistura de O_2 e CO_2 (95:5) através de um oxigenador de membrana e simultaneamente aquecido a 37 °C. Isolamento e canulação do fígado foram feitos sob anestesia de pentobarbital sódico (50 mg/Kg). Dois modos de perfusão foram utilizados: a) anterógrado (veia porta + artéria hepática → veia hepática); b) retrógrado (veia hepática + artéria hepatica → veia porta).

As seguintes substâncias foram dosadas no perfusado efluente através de técnicas enzimático/espectrofotométricas: glicose, lactato, piruvato, glutamina, glutamato, uréia, amônia e alanina. O consumo de oxigênio foi monitorado polarograficamente. A produção de $^{14}CO_2$ a partir de [U-^{14}C]glutamina foi medida por espectroscopia de cintilação líquida após a captação do $^{14}CO_2$ em feniletilamina.

Basicamente dois protocolos experimentais foram seguidos: [a] quatorze minutos após o início da infusão arterial de amônia (até 9.5 μmol min^{-1} g^{-1}) em perfusão anterógrada ou retrógrada, os precursores lactato (45 min) ou piruvato (25 min) foram adicionalmente infundidos na artéria hepática em níveis saturantes (21 e 8.5 μmol min^{-1} g^{-1}, respectivamente); [b] quatorze minutos após o início da infusão de glutamina (0.6 mM) nas veias porta (perfusão anterógrada) ou hepática (perfusão retrógrada), a infusão arterial de amônia (até 9.5 μmol min^{-1} g^{-1}) foi feita por mais 45 minutos. Os fluxos metabólicos decorrentes da transformação dos substratos foram normalizados por divisão pelo espaço celular acessível através da artéria hepática em perfusão anterógrada (J_{ant} — média ponderada de todo o fígado) e retrógrada (J_{ret} — somente células periportais) e expressos como μmol min^{-1} ml^{-1}.

RESULTADOS — 1) A transformação da glutamina em concentrações fisiológicas (0.6 mM) ocorreu apenas na presença de amônia. Altas concentrações arteriais de glutamina foram inibitórias para a sua transformação.

2) Para a gliconeogênese, predominância periportal foi encontrada com lactato (i.e., $J_{ret} > J_{ant}$) na ausência de amônia, mas não com piruvato ($J_{ret} = J_{ant}$); na presença de amônia, predominância periportal foi encontrada com glutamina e piruvato; a predominância periportal com lactato tendeu a desaparecer com concentrações crescentes de amônia.

3) Os incrementos no consumo de O_2 comportaram-se de modo semelhante à gliconeogênese com piruvato + amônia e glutamina + amônia; predominância periportal ocorreu com lactato + amônia para todas as concentrações de amônia.

4) A ureogênese com lactato + amônia revelou predominância em células à jusante da zona periportal ($J_{ant} > J_{ret}$); ausência de predominância ($J_{ant} \approx J_{ret}$) foi encontrada com glutamina + amônia; a ureogênese com piruvato + amônia revelou predominância periportal apenas com altas concentrações de amônia.

5) A produção de alanina foi uma função saturável da velocidade de infusão de amônia; apresentou predominância perivenosa com lactato e piruvato como precursores de carbono para todas as velocidades de infusão de amônia.

6) Incertezas sobre os espaços celulares nos quais a glutamina e o glutamato são produzidos, não autorizam o cálculo de J_{ant} e J_{ret}. Quando expressa como μmol min^{-1} g^{-1}, a produção de glutamato em perfusão retrógrada foi sempre menor do que em perfusão anterógrada. A produção de glutamina de piruvato + amônia também foi sempre menor em perfusão

retrógrada. Com lactato + amônia, a produção de glutamina em perfusão retrógrada aumentou linearmente com a velocidade de infusão de amônia, chegando a superar os valores obtidos em perfusão anterógrada.

7) A produção de $^{14}CO_2$ de [U-^{14}C]glutamina, uma medida do metabolismo total da glutamina, revelou uma clara predominância periportal ($J_{ret} > J_{ant}$).

8) Assumindo cinética de Michaelis-Menten, uma análise numérica dos fluxos metabólicos determinados pela microcirculação hepática revelou-se possível para as velocidades de produção de alanina e uréia a partir de piruvato + amônia como função da velocidade de infusão de amônia. A análise permitiu obter parâmetros cinéticos (constantes de semi-saturação e velocidades máximas), para duas diferentes regiões ao longo do ácino hepático e também as frações do fluxo arterial que atingem as confluências artério-portais intra- e pré-sinusoidais que foram, respectivamente, 58,6 e 41,4%.

CONCLUSÕES E DISCUSSÃO — 1) Os resultados obtidos no presente estudo confirmam a noção geral de que a gliconeogênese e o consumo de oxigênio a ela associado tendem a predominar na região periportal do parênquima hepático. No entanto, os dados também revelam que esta predominância não é incondicional. Além da ausência de zoneamento quando um estado oxidado é induzido pela infusão de piruvato, ficou também evidente que o zoneamento pode ser abolido pela amônia quando lactato é o substrato ou induzida pela amônia quando piruvato é o substrato.

2) A predominância da síntese de alanina a partir de lactato e piruvato + amônia na região perivenosa é um fenômeno inesperado em virtude da conhecida predominância da alanina aminotransferase na região periportal. Esta observação é uma clara indicação de que dados sobre atividade ou expressão de enzimas por si só não podem ser extrapolados incondicionalmente à célula em funcionamento. Neste caso específico está claro que, sob condições celulares, a atividade da alanina aminotransferase não está de acordo com as medidas de atividade, geralmente feitas sob condições artificiais.

3) Predominância da ureogênese na região periportal não foi encontrada, exceto para condições de altas concentrações de amônia mais um estado oxidado induzido por piruvato. A ureogênese, portanto, apresenta-se como outro exemplo de medidas de atividade e expressão de enzimas que não permitem prever a verdadeira distribuição da atividade sob condições celulares.

4) A produção de glutamina na região periportal a partir de lactato ou piruvato + amônia, similar à já relatada produção de glutamina a partir de alanina, é muito improvável que esteja ocorrendo em hepatócitos periportais. Esta sintese ocorre mais provavelmente nas células de Kupffer e endoteliais da região periportal, nas quais a glutamina sintase já foi detectada.

5) A visão atual do sistema hepático de detoxificação de amônia propõe que a pequena fração de células perivenosas sintetizadoras de glutamina remove uma pequena fração da amônia que escapa da ureogênese nas células periportais. No entanto, mesmo quando transformando apenas glutamina, a ureogênese está mais ou menos uniformemente distribuída no parênquima hepático. Assim, provavelmente, a ureogênese é um importante detoxificador de amônia também nas células perivenosas, com exceção da pequena fração destas células que não possui a carbamoil-fosfato sintase.

ABSTRACT

INTRODUCTION AND AIMS — There is general agreement that in the liver the metabolic activity is heterogeneously distributed along the hepatic acini. Some routes predominate in periportal and others in perivenous cells. Evidence favouring this view has been obtained in enzyme distribution studies, flux measurements in isolated perivenous and periportal hepatocytes and in the perfused rat liver and, more recently, in a study of differential gene expression in periportal and perivenous hepatocytes. In spite of this general agreement and of the numerous studies that have been done, there are several aspects still awaiting a more detailed investigation. Evidence indicates that zonation presents some degree of flexibility in that it may suffer considerable alterations when the metabolic conditions are changed. Furthermore, conflicting observations are not uncommon, especially when reciprocally regulated fluxes are analyzed as for example the carbon and nitrogen fluxes involving amino acids and ammonia. The most common discrepancies are those between enzyme activity or gene expression measurements and the actual metabolic fluxes in the living cell. For example, urea production due to alanine transformation in the perfused rat liver was found to be uniformly distributed over the liver parenchyma. This observation is unexpected by virtue of the finding that the expression of several key enzymes of the urea cycle predominates in the periportal region. Moreover, several investigators have found that the alanine aminotransferase activity predominates in periportal hepatocytes. Higher rates of gluconeogenesis from alanine and the associated increases in oxygen uptake were indeed found in the periportal zone. Parallel observations strongly suggest, however, that this periportal predominance is not primarily caused by the periportal predominance of the alanine aminotransferase activity. It is more likely the consequence of the general periportal predominance of the gluconeogenic pathway.

The results on the hepatic zonation of alanine metabolism discussed above raise the question about the true functional distribution of alanine aminotransferase and the urea cycle along the hepatic acini. An alternative way of analyzing the functional distributions of alanine aminotransferase and the urea cycle along the hepatic acini would be to measure alanine and urea productions from precursors such as lactate or pyruvate plus ammonia. These are precisely the main purposes of the present work in which these and several additional parameters related to the carbon and the nitrogen fluxes were measured in the bivascularly perfused rat liver. It has been shown by previous work that infusion of substrates via the hepatic artery in retrograde perfusion allows to reach selectively periportal cells in contrast to the same infusion in antegrade perfusion where all cells are reached. This particularity provides a suitable method for investigating metabolic zonation in the intact rat liver. The results should contribute for a more extensive understanding of the hepatic zonation of the carbon and nitrogen fluxes derived from the ammonia, lactate, pyruvate and glutamine transformations under several conditions.

METHODS — Male Wistar rats, weighing between 180 and 220 g, *ad libitum* fed with a standard chow diet, were used. Food was withdrawn 24 hours prior to the liver perfusion experiments. The liver was perfused bivascularly in the non-recirculating mode. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of O_2 and CO_2 (95:5) by means of a membrane oxygenator and simultaneously heated to 37 °C. Isolation and cannulation of the liver were done under sodium pentobarbital anesthesia (50 mg/Kg). Two perfusion modes were employed: a) antegrade (portal vein + hepatic artery → hepatic vein); b) retrograde (hepatic vein + hepatic artery → portal vein).

The following substances were assayed in the effluent perfusate by means of enzymatic/spectrophotometric techniques: glucose, lactate, pyruvate, glutamine, glutamate, urea, ammonia and alanine. Oxygen uptake was monitored polarographically. The production of $^{14}CO_2$ from [^{14}C]glutamine was measured by liquid scintillation spectroscopy after trapping the $^{14}CO_2$ in phenylethylamine.

Basically two the experimental protocols were employed: [a] fourteen minutes after initiation of the arterial ammonia infusion (up to 9.5 μmol min^{-1} g^{-1}) in antegrade or retrograde perfusion, the precursors lactate (45 min) or pyruvate (25 min) were additionally infused into the hepatic artery at saturing levels (21 and 8.5 μmol min^{-1} g^{-1}, respectively); [b] fourteen minutes after initiation of glutamine infusion (0.6 mM) into the portal (antegrade) or hepatic vein (retrograde), arterial ammonia infusion (up to 9.5 μmol min^{-1} g^{-1}) was done for additional 45 minutes. The metabolic fluxes due to substrate transformations were normalized by division through the cell space that is accessible via the hepatic artery in antegrade (J_{ant} — weighted mean over the whole liver) and retrograde perfusion (J_{ret} — only periportal cells) and expressed as μmol min^{-1} ml^{-1}.

RESULTS — 1) The transformation of glutamine at physiological concentrations (0.6 mM) occurred only in the presence of ammonia. High arterial glutamine concentrations were inhibitory for its own transformation.

2) For gluconeogenesis, periportal predominance was found with lactate (i.e., $J_{ret} > J_{ant}$) in the absence of ammonia, but not with pyruvate ($J_{ret} = J_{ant}$); in the presence of ammonia, periportal predominance was found with glutamine and pyruvate; periportal predominance with lactate tended to vanish with increasing ammonia concentrations.

3) The oxygen uptake increments behaved similarly to gluconeogenesis in the case of pyruvate + ammonia and glutamine + ammonia; periportal predominance was found with lactate + ammonia for all ammonia concentrations.

4) Ureogenesis from lactate + ammonia showed predominance of cells situated downstream to the periportal region ($J_{ant} > J_{ret}$); no predominance ($J_{ant} \approx J_{ret}$) was found with glutamine + ammonia; ureogenesis from pyruvate plus ammonia presented periportal predominance only at high ammonia concentrations.

5) Alanine production was a saturable function of the ammonia infusion rate and showed a clear predominance in the perivenous region with both lactate and pyruvate as carbon precursors and for all ammonia infusion rates.

6) Uncertainties about the cell spaces in which glutamine and glutamate productions occur, do not authorize the calculation of J_{ant} and J_{ret} values. When expressed as μmol min^{-1} g^{-1}, glutamate production in retrograde perfusion was always less than that in antegrade perfusion. Glutamine production from pyruvate + ammonia was also always less pronounced in

retrograde perfusion. With lactate + ammonia, glutamine production in retrograde perfusion increased linearly with the ammonia infusion rate and exceeded that in antegrade perfusion at the highest infusion rate.

7) The production of $^{14}CO_2$ from [^{14}C]glutamine, a measure of the total glutamine metabolism, showed a clear periportal predominance ($J_{ret} > J_{ant}$).

8) Assuming Michaelis-Menten kinetics a numerical model analysis of the metabolic fluxes based on the hepatic microcirculation was possible for the alanine and urea production rates from pyruvate plus ammonia as a function of the ammonia infusion rate. The analysis allowed to obtain kinetic parameters (half-saturation constants and maximal rates) for two different regions along the hepatic acinus in addition to the fractions of the arterial flow reaching the intra- and presinusoidal arterio-portal confluences, which were 58.6 and 41.4%, respectively.

CONCLUSIONS AND DISCUSSION — 1) The results obtained in the present study confirm the general notion that gluconeogenesis and the associated oxygen uptake tend to predominate in the periportal region of liver parenchyma. However, the data also revealed that this predominance is not unconditional. Besides the absence of zonation when an oxidized state is induced by pyruvate infusion, it became also evident that zonation can be abolished by ammonia when lactate is the substrate or induced by ammonia when pyruvate is the substrate.

2) The predominance of alanine synthesis from lactate and pyruvate + ammonia in the perivenous region is an unexpected phenomenon by virtue of the reported predominance of alanine aminotransferase in the periportal region. This observation is a clear indication that data on enzyme activity or expression alone cannot be extrapolated unconditionally to the living cell. In this specific case it is apparent that under real cellular conditions the activity of the alanine aminotransferase does not conform with the activity measurements generally done under artificial conditions.

3) No predominance of ureogenesis in the periportal region was found, except for conditions of high ammonia concentrations plus oxidized conditions induced by pyruvate. Ureogenesis, thus, provides another example of enzyme activity and expression measurements that do not allow to predict the true activity under cellular conditions.

4) The production of glutamine in the periportal region from lactate or pyruvate + ammonia, similar to the reported glutamine production from alanine, is unlikely to occur in periportal hepatocytes. This synthesis occurs more likely in periportal Kupffer and endothelial cells, in which the glutamine synthase has been detected.

5) The current view of the hepatic ammonia-detoxifying system proposes that the small perivenous fraction of glutamine synthesizing perivenous cells removes a minor fraction of ammonia that escapes from ureogenesis in periportal cells. However, even when transforming solely glutamine, ureogenesis is more or less uniformly distributed over the whole liver parenchyma and, most probably, ureogenesis is also an important ammonia-detoxifying mechanism in the perivenous region except in the small fraction of cells deprived from carbamoyl-phosphate synthase.

Flexibility of the hepatic zonation of carbon and nitrogen fluxes linked to lactate and pyruvate transformations in the presence of ammonia

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Running tittle: Zonation of carbon and nitrogen fluxes in the liver.

Abstract

It has been proposed that key enzymes of ureogenesis and the alanine aminotransferase activity predominate in periportal hepatocytes. However, ureogenesis from alanine, when measured in the perfused liver, did not show periportal predominance and even the release of the direct produts of alanine transformation, lactate and pyruvate, was higher in perivenous cells. An alternative way of analyzing the functional distributions of alanine aminotransferase and the urea cycle along the hepatic acini would be to measure alanine and urea productions from precursors such as lactate or pyruvate plus ammonia. In the present work these aspects were investigated using the bivascularly perfused rat liver. The results of the present study confirm that gluconeogenesis and the associated oxygen uptake tend to predominate in the periportal region. Alanine synthesis from lactate and pyruvate + ammonia, however, predominated in the perivenous region. Furthermore, no predominance of ureogenesis in the periportal region was found, except for conditions of high ammonia concentrations plus oxidizing conditions induced by pyruvate. These observations corrobobate the view that data on enzyme activity or expression alone cannot be extrapolated unconditionally to the living cell. The current view of the hepatic ammonia-detoxifying system proposes that the small perivenous fraction of glutamine synthesizing perivenous cells removes a minor fraction of ammonia that escapes from ureogenesis in periportal cells. However, since urea synthesis occurs at high rates in all hepatocytes with the possible exclusion of those cells not possessing carbamoyl-phosphate synthase, it is probable that ureogenesis is equally important as an ammonia-detoxifying mechanism in the perivenous region.

Introduction

There is general agreement that in the liver the metabolic activity is heterogeneously distributed along the hepatic acini. Some routes predominate in periportal and others in perivenous cells. Evidence favouring this view has been obtained in enzyme distribution studies (19,20), flux measurements in isolated perivenous and periportal hepatocytes (30) and in the perfused rat liver (6,7,20) and, more recently, in a study of differential gene expression in periportal and perivenous hepatocytes (9). In spite of this general agreement and of the numerous studies that have been done, there are several aspects still awaiting a more detailed investigation. Evidence indicates that zonation presents some degree of flexibility in that it may suffer considerable alterations when the metabolic conditions are changed (18). Furthermore, conflicting observations are not uncommon, especially when reciprocally regulated fluxes are analyzed as for example the carbon and nitrogen fluxes involving amino acids and ammonia. The most common discrepancies are those between enzyme activity or gene expression measurements and the actual metabolic fluxes in the living cell. For example, urea production due to alanine transformation in the perfused rat liver is uniformly distributed over the liver parenchyma and for this reason the molar ratio ureogenesis/gluconeogenesis is smaller in periportal cells when compared to perivenous cells (6). The latter observations are unexpected by virtue of the finding that the expression of several key enzymes of the urea cycle predominates in the periportal region (9,15). Moreover, several investigators have found that the alanine amino-transferase activity predominates in periportal hepatocytes (1,5,10, 27,30,33). This leads to expect a more intense alanine metabolism in the periportal zone. In fact, higher rates of gluconeogenesis from alanine and the associated increases in oxygen uptake were found in the perfused rat liver (6). Parallel observations strongly suggest, however, that this periportal predominance is not primarily caused by the periportal predominance of the alanine aminotransferase activity. It is more likely the consequence of the general periportal predominance of the gluconeogenic pathway, a conclusion derived from the observation that the overflows of pyruvate and lactate, the first products of alanine transformation, were considerably smaller in periportal cells (6).

The results on the hepatic zonation of alanine metabolism discussed above raise the question about the true functional distribution of alanine aminotransferase and the urea cycle along the hepatic acini. An alternative way of analyzing the

functional distributions of alanine aminotransferase and the urea cycle along the hepatic acini would be to measure alanine and urea productions from precursors such as lactate or pyruvate plus ammonia. These are precisely the main purposes of the present work in which alanine and urea productions from these precursors were measured in the bivascularly perfused rat liver in antegrade and retrograde perfusion. It has been shown by previous work that infusion of substrates via the hepatic artery in retrograde perfusion allows to reach selectively periportal cells in contrast to the same infusion in antegrade perfusion, a procedure in which all cells are reached. This particularity provides a suitable method for investigating metabolic zonation in the intact rat liver without the disturbances inherent to cells subject to an entirely artificial environment (6,7,28). Besides alanine and urea productions, several additional parameters related to the carbon and the nitrogen fluxes were measured at different ammonia concentrations and under different redox states, in order to obtain a more complete picture about the zonation of these fluxes under various conditions.

Materials and methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the metabolite assays were purchased from Sigma Chemical Co. (St Louis, USA). All standard chemicals were from the best available grade (>99.5 % purity) and were purchased from Merck (Darmstadt, FRG), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

Bivascular liver perfusion

Male albino rats (Wistar), weighing 180-220 g, were fed *ad libitum* with a standard laboratory diet (Purina®). Food was withdrawn 24 hours prior to the liver perfusion experiments. For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). All experiments were done in accordance with the recommendations of the institutional ethics committee for animal experimentation.

Hemoglobin-free, non-recirculating bivascular liver perfusion was performed either in the antegrade (entry via the portal vein plus hepatic artery and exit via the hepatic vein) or in the retrograde mode (entry via the hepatic vein plus hepatic artery and exit via the portal vein). The surgical procedure was described elsewhere (32). *In situ* perfusion was carried out, the flow being provided by two peristaltic pumps. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine-serum albumin, saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment (37°C). The portal flow was adjusted between 28 and 32 ml/min and the arterial flow between 2 and 3 ml/min. All perfusion experiments were initiated in the antegrade mode. Retrograde perfusion was established by changing the direction of flow at 15-20 minutes before initiating sampling of the effluent perfusate.

In all perfusion experiments livers from fasted rats were used so that glycogenolysis and glycolysis from endogenous sources was minimal (2). The substrates were lactate (21 μmol min^{-1} g^{-1}) plus ammonia (up to 9.5 μmol min^{-1} g^{-1}) or pyruvate (8.5 μmol min^{-1} g^{-1}) plus ammonia.

Analytical

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were measured by means of standard enzymatic procedures: glucose, lactate, pyruvate, alanine, glutamate, glutamine, urea and ammonia (3). The oxygen concentration in the outflowing perfusate was monitored continuously, employing a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate (8).

Data treatment and interpretation

The changes in metabolic fluxes caused by lactate plus amonia or pyruvate plus ammonia infusion into the hepatic artery were expressed as μmol min^{-1} (ml accessible cell space)$^{-1}$ in each perfusion mode (12), after subtracting the basal rates. The basal rates are those ones measured before any substrate infusion. This procedure effectively normalizes the responses of the liver and provides a basis for comparison in spite of the different cell spaces that are reached via the hepatic artery in antegrade and retrograde perfusion (see Figure 1). The effective substrate concentrations along the hepatic acinus, however, depend on the distribution of the arterial flow between the pre- and intrasinusoidal confluences, a fact, which must also be taken into account when interpreting the results.

A simple model for the flux responses that can be expected when substrates are infused into the hepatic artery in antegrade or retrograde perfusion has been proposed in a previous work (6). If J_{ret} and J_{ant} are the cell space normalized metabolic fluxes due to the infusion of saturing substrate concentrations into the hepatic artery in retrograde and antegrade perfusion, respectively, the following simple relations are valid:

$$J_{ret} = V_1 \quad [1]$$

$$J_{ant} = V_1 r + V_2(1 - r) \quad [2]$$

V_1 is the maximal rate in the region between the pre- and intrasinusoidal confluence and V_2 the maximal rate in the region situated downstream to the intrasinusoidal confluence (Figure 1); r is the fractional cell volume between the pre- and the intrasinusoidal confluences, which has been determined to be equal to 0.38 for the isolated and hemoglobin-free perfused rat liver (12,13). Since J_{ret} represents the response of periportal cells and J_{ant} the mean response of all cells,

three simple rules can be applied: if $J_{ret} = J_{ant}$, no zonation exists; if $J_{ret} > J_{ant}$, periportal predominance exists; if $J_{ant} > J_{ret}$, the predominance is perivenous. Due to these simple relationships, the gluconeogenic substrates lactate and pyruvate were infused at saturing concentrations. For lactate this corresponds anyway to the physiologic conditions. For pyruvate it is an important experimental condition, i.e., a low NADH/NAD^+ ratio as opposed to the high ratio in the presence of lactate (31). For ammonia, however, the physiologic conditions are normally well below saturation and, for this reason, a wide range of infusion rates must be used if the data are to be appropriately interpreted. In terms of the microcirculatory peculiarities of the liver, responses to ammonia infusion (S_{in}) in the presence of lactate or pyruvate in retrograde (J_{ret}) and antegrade (J_{ant}) perfusion obeying the Michaelis-Menten equation can be described by the following equations (6):

$$J_{ret} = J_{ret_o} + \frac{V_1 S_{in}}{K_1(F_T F_A / F_{AIS}) + S_{in}} \quad [3]$$

$$J_{ant} = J_{ant_o} + \frac{rV_1 S_{in}}{K_1[F_T F_A /(F_A - F_{AIS})] + S_{in}} + \frac{(1-r)V_2 S_{in}}{K_2 F_T + S_{in}} \quad [4]$$

S_{in} represents the rate of ammonia infusion (in μmol min^{-1} gram $liver^{-1}$). J_{ret_o} and J_{ant_o} represent the rates of the metabolic fluxes in the absence of ammonia due to the infusion of saturing levels of lactate or pyruvate into the hepatic artery in retrograde and antegrade perfusion, respectively. F_T is the total flow through the liver (in ml min^{-1} g $liver^{-1}$), F_A is the arterial flow and F_{AIS} the arterial flow that reaches the intrasinusoidal confluence (see Figure 1). K_1 and K_2 are, respectively, the half-saturation concentrations for the region between the pre- and intrasinusoidal confluence and for the region situated downstream to the intrasinusoidal confluence. For identical infusion rates (S_{in}), the combined parameter (S_{in}/F_T) is always greater than ($F_{AIS}S_{in}/F_T F_A$) and, in practical terms, if the experimental J_{ret} is found greater than J_{ant} at sub-saturing conditions (i.e., $J_{ret} > J_{ant}$), this clearly indicates periportal predominance. And also, if J_{ant} persistently exceeds J_{ret} over a wide concentration range without any tendency of convergence at saturing concentrations (i.e., $J_{ant} >> J_{ret}$), this can be regarded as an indication of perivenous predominance (6).

If the determination of K_1, K_2, V_1 and V_2 is possible, the metabolic fluxes in the region between the pre- and intrasinusoidal confluence (J_1) and in the region that is

situated downstream to the intrasinusoidal confluence (J_2) can be calculated for any ammonia concentration:

$$J_1 = \frac{V_1[NH_3]}{K_1 + [NH_3]} \quad [5]$$

$$J_2 = \frac{V_2[NH_3]}{K_2 + [NH_3]} \quad [6]$$

$J_1 = J_2$ will mean absence of zonation, $J_1 > J_2$ periportal predominance and $J_2 > J_1$ perivenous predominance.

Treatment of data

Statistical analysis of the data was done by means of the Statistica™ program (Statsoft®, 1998). Fitting of equations [3] and [4] to experimental data was performed by means of an iterative non-linear least-squares procedure, using the Scientist® software from MicroMath Scientific Software (Salt Lake City, USA).

Results

Time courses of the responses to ammonia plus lactate or pyruvate

The time course of the responses of the liver in antegrade and retrograde perfusion to arterial ammonia and lactate infusion are illustrated by Figures 2 and 3; Figure 2 shows the changes in oxygen uptake and in the productions of glucose and pyruvate (electron and carbon fluxes) and Figure 3 the changes in urea, glutamine, glutamate and alanine productions (nitrogen fluxes). After a pre-perfusion period (10 minutes) ammonia was initially infused into the hepatic artery during 14 minutes. In the experiments illustrated by Figures 2 and 3 the rate of ammonia infusion into the hepatic artery was equal to 2.3 μmol min^{-1} g^{-1}, which can be expected to produce a final mean sinusoidal concentration of 0.5 mM. Lactate infusion was initiated at 24 minutes perfusion time and continued until the end of the experiment (70 minutes). The lactate infusion rate was equal to 21 μmol min^{-1} g^{-1}, which should produce a final mean sinusoidal concentration of 5 mM, which is saturing for lactate metabolism (35). Except for oxygen uptake, the basal rates (before substrate infusion) were all low. The infusion of ammonia produced significant increases in urea and glutamine productions and small increments in oxygen uptake in both perfusion modes, antegrade and retrograde. Lactate infusion in addition to ammonia produced much more pronounced changes, with initially accelerated increases, followed by phases of stabilization. All changes were smaller in retrograde perfusion, an expected event because the cell space which is accessible via the hepatic artery in retrograde perfusion corresponds to only 38% of that one accessible in antegrade perfusion (12,13). The extent of the changes caused by lactate in antegrade and retrograde perfusion, however, were not the same for all parameters. For glucose production and oxygen uptake, for example, the final difference between antegrade and retrograde perfusion was relatively small (Figure 2). The urea and alanine productions, on the other hand, increased to a relatively small extent in retrograde perfusion (Figure 3).

The experimental protocol and the time courses of the experiments in which pyruvate was used as the carbon source are illustrated by Figures 4 and 5. Figure 4 shows the changes in oxygen uptake and in the productions of glucose and lactate (electron and carbon fluxes) and Figure 5 the changes in urea, glutamine, glutamate and alanine productions (nitrogen fluxes). After a pre-perfusion period of 10 minutes, ammonia was initially infused into the hepatic artery during 14

minutes. Pyruvate infusion was initiated at 24 minutes perfusion time and continued until the end of the experiment (50 minutes). The pyruvate infusion rate of 8.5 μmol min^{-1} g^{-1} should produce a mean final sinusoidal concentration around 2 mM, which is a saturing for pyruvate metabolism (35). The responses to pyruvate were similar to the responses to lactate. The main differences to be remarked are that the increments in glucose production and oxygen uptake were smaller, but the increments in alanine and glutamate production were considerably higher, especially in the antegrade mode of perfusion.

Metabolic fluxes in antegrade and retrograde perfusion corrected for the accessible cell spaces

The experiments shown in Figures 2 to 5 were obtained with an ammonia infusion rate of 2.3 μmol min^{-1} g^{-1}. They were repeated without previous ammonia infusion (zero ammonia) and with two additional ammonia infusion rates, namely 4.6 and 9.5 μmol min^{-1} g^{-1}. Since different cellular spaces are supplied with substrates infused into the hepatic artery in antegrade and retrograde perfusion (see Figure 1), normalization is essential for conclusions about zonation. The cellular spaces accessible in each perfusion mode have already been determined in previous work (12,13). When using these cellular spaces for normalization it is implicitly assumed that the given metabolites are in fact being produced in these spaces. This is a reasonable assumption for glucose and alanine production and also for oxygen uptake. Urea production can also be analyzed in this way because more than 90% of the hepatocytes express the enzymes of the urea cycle (15,16). Normalization of the metabolic fluxes consists in dividing each metabolic flux in the presence of ammonia and lactate or pyruvate by the corresponding accessible cell space after subtracting the basal rates. Basal rates are those ones measured before ammonia infusion. It is thus assumed that the increments caused by lactate or pyruvate plus ammonia are due solely to the transformation of these substrates in the corresponding cell space. In all cases the values at the end of the substrate infusion period (50 minutes for pyruvate and 70 minutes for lactate) were used. The results are summarized in Figures 6 and 7 for each metabolic flux and condition. All data were represented against the rate of ammonia infusion.

As presented in the Materials and Methods section, the normalized increments in retrograde perfusion (J_{ret}) reflect the periportal region, whereas the increments in antegrade perfusion (J_{ant}) correspond to a weighted mean over the whole liver

parenchyma. Figure 6A reveals higher rates of glucose production from lactate in retrograde perfusion at low ammonia concentrations. Since the rate of lactate infusion produced saturing concentrations of this substrate, the observation that $J_{ret} > J_{ant}$ also means predominance of gluconeogenesis in the periportal region. This corroborates previous observations (7). Confirming previous notions, ammonia inhibited glucose production (29,34), but its action was more pronounced in retrograde perfusion, so that the difference in J_{ret} and J_{ant} diminished progressively as the ammonia infusion was increased. At high ammonia infusion rates the difference between J_{ret} and J_{ant} vanished. The inhibitory action of ammonia on gluconeogenesis from lactate is, thus, stronger in the periportal region. For the oxygen uptake increment caused by lactate (Figure 6C), the situation $J_{ret} > J_{ant}$ was maintained over the whole range of ammonia infusion, meaning thus periportal predominance of the increment in oxygen uptake. Confirming a previous communication (7), glucose production from saturing pyruvate concentrations was similar in periportal and perivenous cells in the absence of exogenously added ammonia, as revealed by Figure 6B. With the increasing rates of ammonia infusion, glucose production was inhibited, with a stronger effect in antegrade perfusion. It is worth to remark that this is opposite of what occurred with gluconeogenesis from lactate. The result was that at high rates of ammonia infusion, J_{ret} was superior to J_{ant}, meaning a shift toward periportal predominance under these conditions. The oxygen uptake increments due to pyruvate (Figure 8D) behaved similarly to glucose production: no difference between J_{ret} and J_{ant} at low rates of ammonia infusion, but a clear tendency toward periportal predominance at high infusion rates.

Urea production with lactate plus ammonia (Figure 6A) was a saturing function of the ammonia infusion rate in both antegrade and retrograde perfusion. For the whole range of ammonia infusion rates, J_{ant} was superior to J_{ret}, meaning thus predominance in cells situated downstream to the periportal region. Alanine productions from lactate plus ammonia (Figure 6C) in antegrade and retrograde perfusion were saturing functions of the ammonia infusion rate with a kind of "substrate inhibition" phenomenon at the highest infusion rate of ammonia. Perivenous predominance is clear, however, due to the fact that J_{ant} was always superior to J_{ret} for the whole range of ammonia infusion rates. The increments in urea production with pyruvate plus ammonia revealed clear saturing functions of the ammonia infusion rate (Figure 7B). J_{ret} and J_{ant} were only slightly different, with a clear tendency of equalizing at high rates of ammonia infusion. By simple

inspection it is difficult to conclude about zonation, a more precise model analysis being indispensable (see below). Figure 7D shows alanine production in antegrade and retrograde perfusion as saturing functions of the ammonia infusion rate; J_{ant} was clearly superior to J_{ret} for the whole range of ammonia infusion rates, meaning perivenous predominance.

Glutamate and glutamine productions as a function of the ammonia infusion rates

It is not advisable to normalize glutamine production in terms of the whole cell spaces tat are accessible via the hepatic artery because this compound is produced by a relatively small number of cells, probably only by hepatocytes immediately surrounding the hepatic venules (16), by Kupffer cells (4) and endothelial cells (24). For this reason the production of these two metabolites was simply expressed as μmol per minute per gram wet liver weight and represented against the ammonia infusion rates. The results are shown in Figure 8. The rates of glutamate release were low. There was no defined increment upon ammonia infusion, except when pyruvate was the substrate in antegrade perfusion (Figure 8B). Glutamate production in antegrade perfusion was always superior to that in retrograde perfusion. Glutamine production from lactate + amonia was a saturable function of the ammonia infusion rate in antegrade perfusion and increased linearly with the infusion rate in retrograde perfusion (Figure 8C). Surprinsingly, this difference in behaviour produced higher rates of glutamine production in retrograde when compared to antegrade perfusion at the highest rate of ammonia infusion. Glutamine production from pyruvate + ammonia, however, was always less pronounced in retrograde perfusion (Figure 8D).

Model analysis

Interpretation of the data in Figures 6 and 7 can be refined by model analysis which takes into account the different substrate concentrations in the two regions of the sinusoidal bed according to the scheme shown in Figure 1. Equations [3] and [4] can be fitted to the experimental data in order to determine the various parameters. These equations represent Michaelian saturation functions, and they will produce reliable parameters only if the experimental data also conform to the same function. Visual inspection of the various relationships in Figures 6 and 7

reveals that the curves of urea and alanine productions from pyruvate versus the ammonia infusion rates (Figures 7B and 7D) are typical saturation curves. Alanine production from lactate (Figure 7C) is not adequate because it presents substrate inhibition and urea production from lactate is pratically saturated with the lowest rate of ammonia infusion. Based on these criteria the data on alanine and urea productions from ammonia plus pyruvate were chosen for the analysis and equations [3] and [4] were fitted simultaneously to the experimental data. A non-linear least squares procedure was utilized and the results are illustrated by Figure 9. The optimized parameters that were obtained from the fitting procedure are listed in the legend to Figure 9. Actually two sets of equations were used, one for alanine and the other one for urea. In the legend to Figure 9 the kinetic parameters for alanine and urea can be distinguished by their different subscripts, namely K_{A1}, K_{A2}, V_{A1} and V_{A2} for alanine production and K_{U1}, K_{U2}, V_{U1} and V_{U2} for urea production. As explained in the Materials and Methods section, the subscript 1 refers to the region between the presinusoidal and intrasinusoidal arterio-portal confluences and the subscript 2 to the region situated downstream to the intrasinusoidal confluence (Figure 1). Figure 9 reveals good agreement between experiment and theory. The computed arterial flow that reaches the intrasinusoidal confluence (F_{AIS}, 0.205 ml min^{-1} g^{-1}) is realistic when compared to the total experimental arterial flow (0.35 ml min^{-1} g^{-1}). The maximal rates for alanine production in both regions are different, the V_{A2}/V_{A1} ratio of 2.33 fully corroborating the conclusion that alanine production is faster in the perivenous region. The apparent Michaelis constants for alanine production are in the milimolar range with a K_{A2}/K_{A1} ratio of 0.68. For urea production, the maximal rates are favouring the periportal region with a V_{U2}/V_{U1} ratio 0.63. However, the apparent Michaelis constants also differ considerably, with a K_{U2}/K_{U1} ratio of 0.29. This means that predominance of urea production in one of the two regions along the hepatic acinus that can be differentiated by the present experimental approach is a function of ammonia concentration. This prediction can be tested by the simulations shown in Figure 10. These graphs are presenting the saturation curves of alanine and urea productions as functions of the extracellular ammonia concentration for the regions along the hepatic acinus that can be differentiated by the present experimental approach. The simulations were done by calculating Michaelian functions with the kinetic parameters obtained in the fitting procedure illustrated by Figure 9. As expected, for alanine production perivenous predominance is clear for the whole range of ammonia concentration. For

ureogenesis, however, the distribution over the liver parenchyma is a function of the ammonia concentration: the perivenous predominance at low ammonia concentrations shifts progressively towards periportal predominance at high concentrations.

Discussion

The method employed in the present work was originally proposed as an advantageous alternative for studying metabolic zonation (Pang et al., 1988). The main advantage is the maintenance of the microcirculation and the organ integrity, what means also maintenance of intercellular cooperation. The recent proposition of a simple model integrating the main microcirculatory features and the differential action of agents and subtrates along the hepatic acinus has facilitated the interpretation (6). This model, which has been adapted to the particular experimental protocol utilized in the present work, deals basically with saturing phenomena which can be complex functions of the rates of substrate or inhibitor infusion. Simulation frequently provides a useful basis for interpretation (6). When the model can be fitted to the experimental data because they conform to the simple Michaelis-Menten relation, however, the method allows a fine resolution of the differential effects of a given substrate along the hepatic acini. In the present work this was the case of urea and alanine productions from pyruvate + ammonia as a function of the ammonia concentration. In addition to the apparent affinity constants for ammonia along the hepatic acinus, fitting of equations [3] and [4] to the data also allowed to detect a shift in the predominance of ureogenesis from perivenous to periportal when the ammonia concentration was raised. Furthermore, the fitting procedure also allowed to obtain an estimate of the arterial flow that reaches the intrasinusoidal confluence (F_{AIS} in equations [3] and [4]; see Figure 1). The actual value that was obtained, 58.5% of the total arterial flow, is quite realistic and confirms previous conclusions from experiments in which the fractional extration of arterially infused ATP was investigated (6,26).

Besides confirming the general notion of a heterogenic distribution of the metabolic activity along the hepatic acinus, the results of the present work also reveal that this distribution is under short-term control and it can even be changed by factors acting in the opposing direction of medium- and long-term factors. As predicted by enzyme activity measurements, gluconeogenesis and the associated oxygen uptake tend to predominate in the periportal region of liver parenchyma. Periportal hepatocytes are in principle better equipped than perivenous hepatocytes for gluconegenesis and oxidative processes. Compared with perivenous hepatocytes, periportal cells have higher activities of key enzymes of gluconeogenesis as well as a greater volume of mitochondria and a greater area of cristae (14,21,22).

However, this periportal predominance of gluconeogenesis and the associated extra oxygen consumption is not unconditional. Besides the absence of zonation when an oxidized state is induced by pyruvate infusion (Bracht et al., 1994), it is also evident from the observations that zonation can be abolished by ammonia when lactate is the substrate or induced by ammonia when pyruvate is the substrate. If one considers that the physiological condition is characterized by high lactate and low pyruvate concentrations and low ammonia concentrations, gluconeogenesis will be predominating in the periportal region although the difference will not be as pronounced as if ammonia were totally absent (7).

The absence of predominance of alanine synthesis from lactate and pyruvate + ammonia in the periportal region is an unexpected phenomenon by virtue of the reported predominance of alanine aminotransferase in the periportal region (1,5,10, 27,30,33). This observation is a clear indication that data on enzyme activity or expression alone cannot be extrapolated unconditionally to the living cell. In this specific case it is apparent that under real cellular conditions the activity of the alanine aminotransferase does not conform to the activities measured under artificial conditions. Most likely the conditions surrounding the alanine aminotransferase in periportal and perivenous cells are substantially different, producing also different net fluxes. Substrate availability is an important factor influencing both the reaction rate and the direction of the net flux. In the direction of alanine synthesis the substrates are pyruvate and glutamate. Pyruvate was constantly infused in the present work so that its availability was not a limiting factor. Glutamate, however, has to be produced by the amination reaction catalyzed by glutamate dehydrogenase, which is an enzyme operating at near-equilibrium conditions. This enzyme is expressed by all hepatocytes and there are observations indicating either a uniform distribution (5) or perivenous predominance (17). Irrespective of the acinar distribution, however, it has been hypothesized that the perivenous glutamate dehydrogenase is highly active in the direction of glutamate production whereas the opposite occurs in periportal hepatocytes (5). The reduced alanine production from lactate or pyruvate + ammonia in the periportal hepatocytes observed in the present work could thus be reflecting a lower flux through glutamate dehydrogenase in the amination direction, generating also a reduced flux of glutamate in the direction of pyruvate transamination. This interpretation should be regarded as one of several possibilities needing confirmation by additional experimental work.

No predominance of ureogenesis in the periportal region was found, except for conditions of high ammonia concentrations plus oxidizing conditions induced by pyruvate. Especially in the presence of lactate and low ammonia concentrations, conditions close to the physiological ones, the mean ureogenic activity of the whole liver parenchyma clearly exceeded that of the periportal cells indicating that ureogenesis is more active in hepatocytes localized downstream to the periportal region. To these observations one should add similar findings of experiments with alanine (6). Periportal predominance of the activity and expression of key enzymes of the urea cycle have been found in several studies (9,15). However, the enzymes of the urea cycle are present in more than 90% of the parenchymal cells. The key-enzyme carbamoyl phosphate synthase, for example, seems to be absent only from those hepatocytes immediately surrounding the hepatic venules (15). The hepatocytes not containing carbamoyl phosphate synthase are precisely those ones containing glutamine synthase. The latter have been estimated to comprise only 7% (16). Most hepatocytes, thus, are perfectly able to synthesize urea and the final ureogenic activity will depend not only on the maximal activity (which is that one detected under artificial conditions) but also on the real cellular conditions in terms of the concentrations of substrates and allosteric regulators. Since there are several enzymes involved, there are also several regulatory possibilities which, in the absence of direct evidence, can only be discussed as more or less likely possibilites. It could be, for example, that the conditions in periportal cells are less favourable than in down-stream localized cells for the production of N-acetyl-glutamate, the key activator of carbamoyl-phosphate synthase (25). This could result, for example, from a reduced amination of 2-oxoglutarate by the glutamate dehydrogenase in periportal hepatocytes, as already mentioned above when discussing the lower rates of alanine production in the periportal region. It should also be remembered that the N-acetyl-glutamate synthase is itself a regulatory enzyme. As such it is subject, to regulatory mechanisms with participation of arginine (23) and ornithine (11), whose concentrations are not necessarily equal along the hepatic acinus. Even for aspartate, which is an essential amine group donnor for the urea cycle, there can be no certainty about its availability along the hepatic acinus. The activity of the aspartate transaminases seems to predominate in periportal cells (1,5), but it is already clear from the example of alanine aminotransferase discussed above that this is not an imperative for enhanced aspartate production. In this particular case, the substrates for aspartate

production, glutamate and oxaloacetate, could be limiting. The first one for the same reasons already discussed above and the second one due to the high gluconeogenic activity, especially in the presence of lactate.

The production of glutamine in the periportal region from lactate or pyruvate + ammonia, similar to the reported glutamine production from alanine, is unlikely to occur in periportal hepatocytes. This synthesis occurs more likely in periportal Kupffer and endothelial cells, in which the glutamine synthase has been detected (4,24). As already stated, it is not advisable to analyze glutamine production in terms of the space normalized parameters J_{ant} and J_{ret} because glutamine is certainly comming from very small cellular spaces when compared to the whole liver. Furthermore, recycling along the hepatic acini is highly probable so that the normalized rates of glutamine production would be poor indicators of an heterogeneous distribution of its production. The results seem thus indicate that Kupffer and endothelial cells localized in the periportal region are perfectly able to produce glutamine as indeed expected from the probable presence of glutamine synthase in these cells (4,24). Alternatively, a fraction of the glutamine production in retrograde perfusion could be the result of a decreased hydrolysis of the glutamine produced in perivenous hepatocytes. This is unlikely, however, if one considers that ammonia is well known for its stimulatory effect on glutaminase (19).

The current view of the hepatic ammonia-detoxifying system proposes that the small perivenous fraction of glutamine synthesizing perivenous cells removes a minor fraction of ammonia that escapes from ureogenesis in periportal cells (20). It certainly continues to be a valid assumption that the perivenous cells immediately surrounding the hepatic venules, which contain glutamine synthase and do not contain carbamoyl phosphate synthase, are able to remove ammonia solely by glutamine synthesis (15,16). However, the fact that ureogenesis, under some specific conditions, can be more active in cells situated downstream to the periportal zone, strongly suggests that this pathway is also an important ammonia-detoxifying mechanism in the perivenous region excepting only the small fraction of cells deprived from carbamoyl-phosphate synthase.

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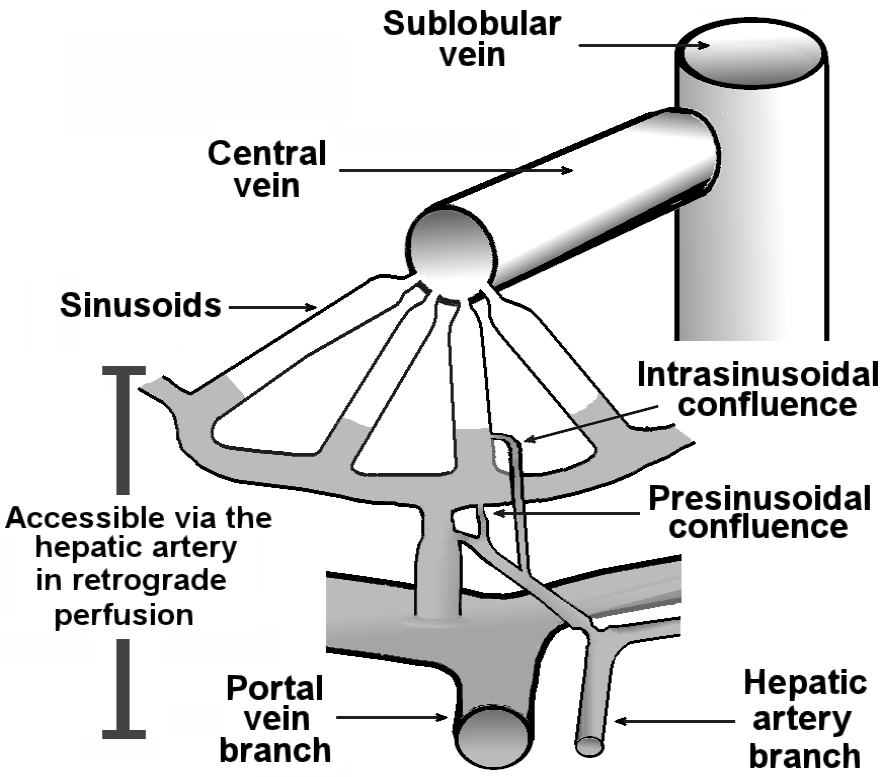

Figure 1. ***Diagram of some important features of the hepatic microcirculation.***

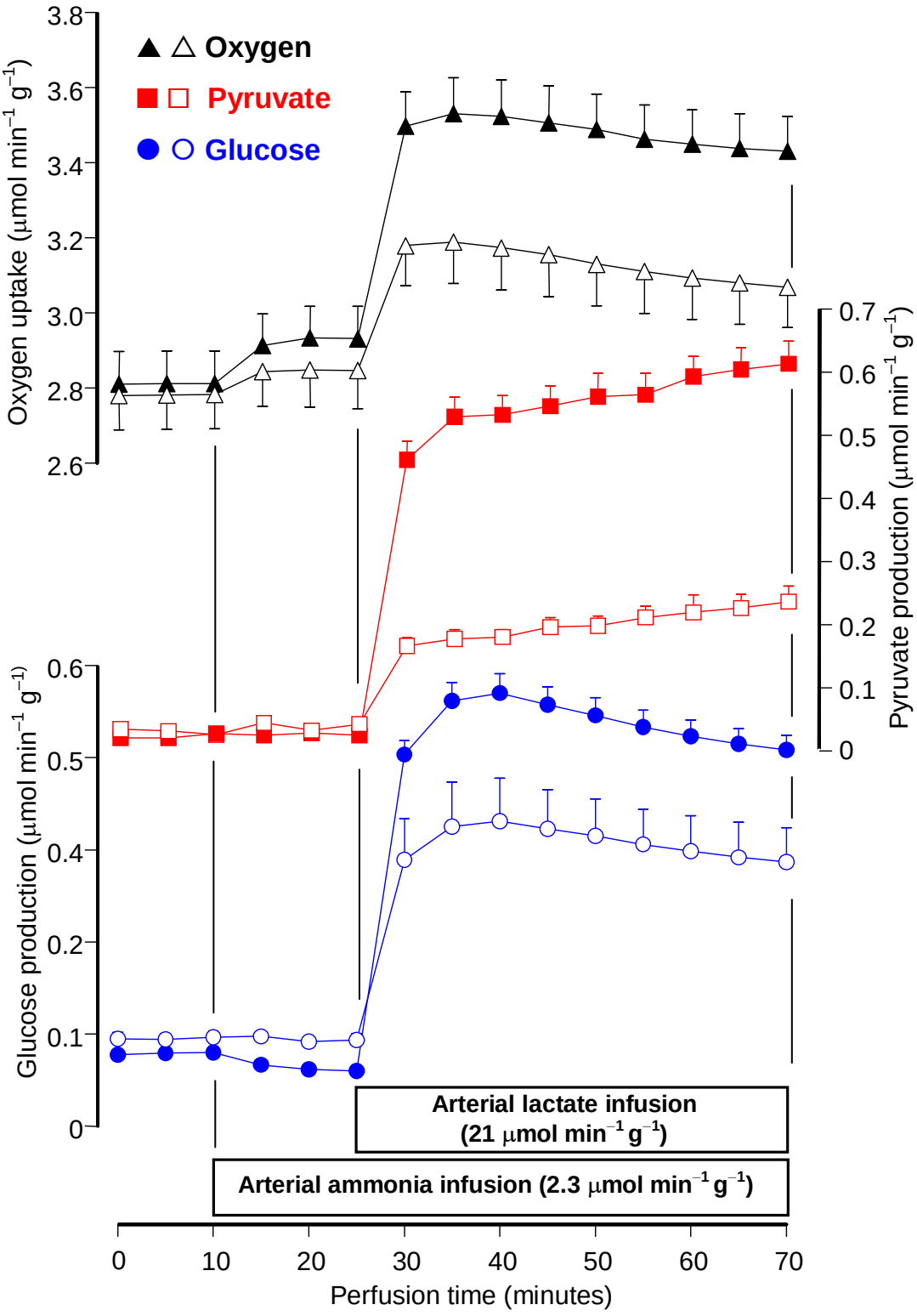

Figure 2. ***The action of arterially infused ammonia and lactate on hepatic carbon fluxes and oxygen uptake in antegrade and retrograde perfusion.*** Livers from fasted rats were perfused as described in Materials and Methods. Lactate and ammonia were infused into the hepatic artery as indicated. Full symbols (▲,■,●) represent the results obtained in antegrade perfusion; empty symbols (△,□,○) represent those ones obtained in retrograde perfusion. Data are from 9 (antegrade) or 6 (retrograde) liver perfusion experiments. Error bars represent mean standard errors.

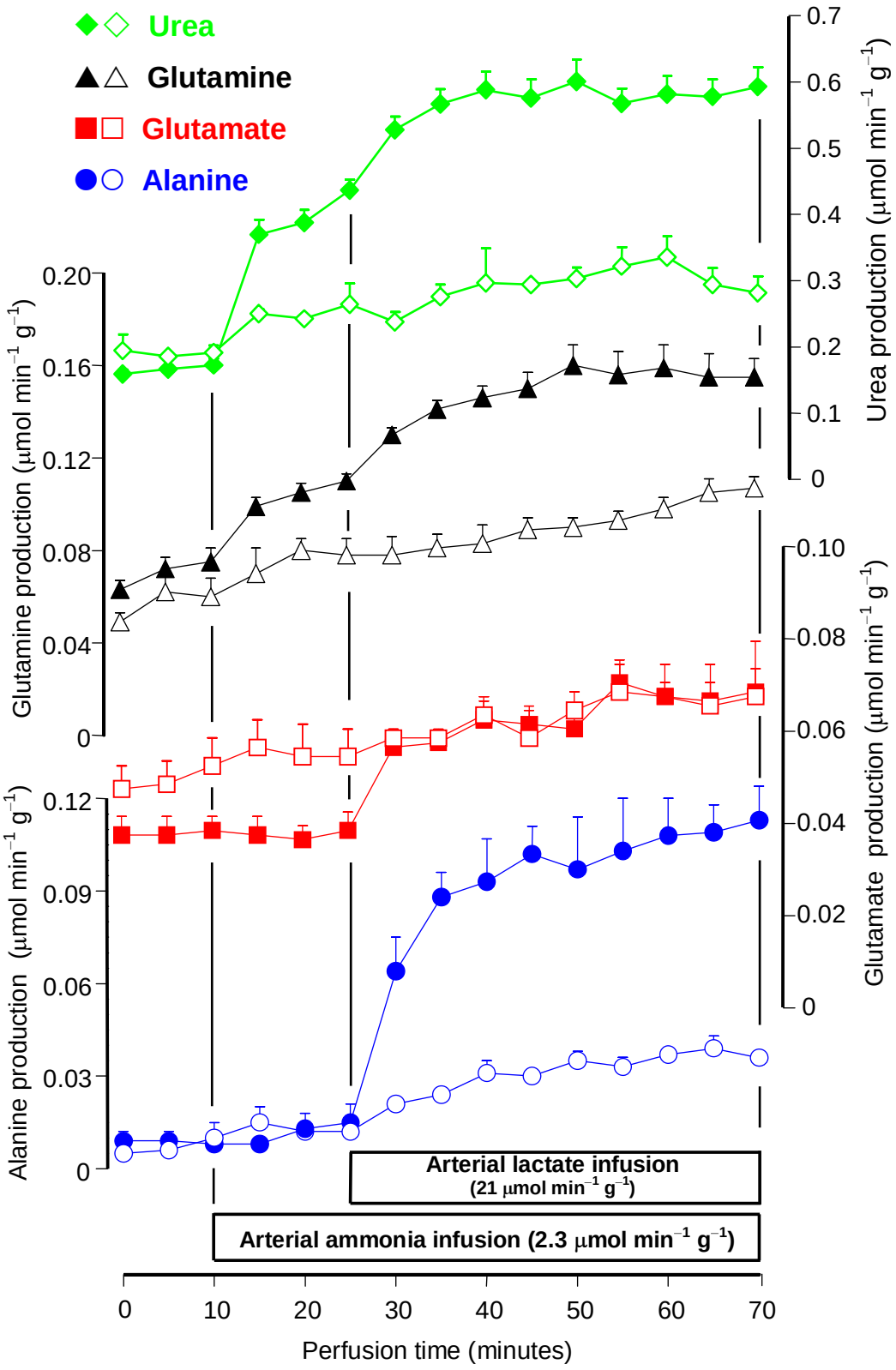

Figure 3. ***The action of arterially infused ammonia and lactate on hepatic nitrogen fluxes in antegrade and retrograde perfusion.*** Livers from fasted rats were perfused as described in Materials and Methods. Lactate and ammonia were infused into the hepatic artery as indicated. Full symbols (◆,▲,■,●) represent the results obtained in antegrade perfusion; empty symbols (◇,△,□,○) represent those ones obtained in retrograde perfusion. Data are from 9 (antegrade) or 6 (retrograde) liver perfusion experiments. Error bars represent mean standard errors.

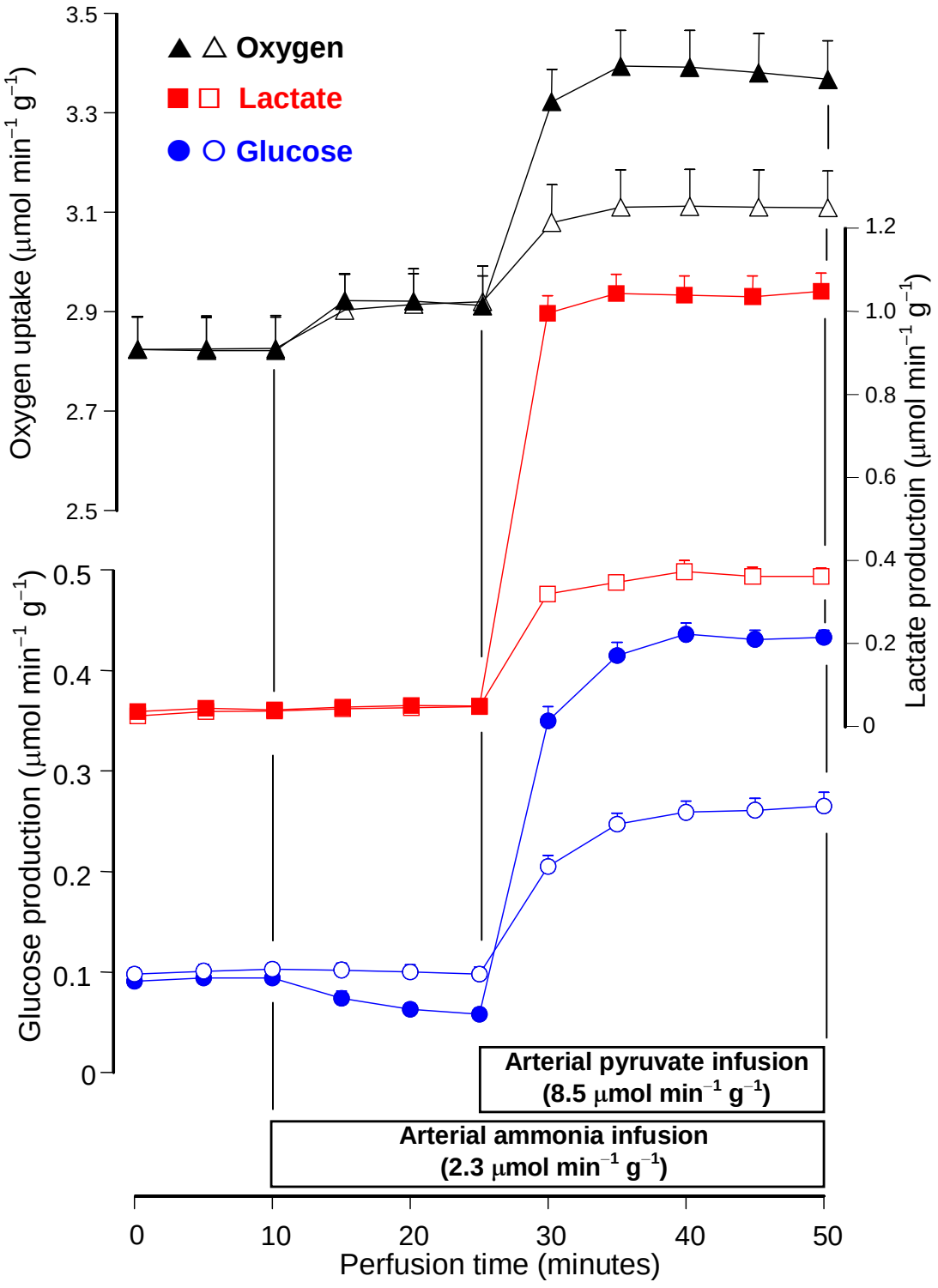

Figure 4. ***The action of arterially infused ammonia and pyruvate on hepatic carbon fluxes and oxygen uptake in antegrade and retrograde perfusion.*** Livers from fasted rats were perfused as described in Materials and Methods. Pyruvate and ammonia were infused into the hepatic artery as indicated. Full symbols (▲,■,●) represent the results obtained in antegrade perfusion; empty symbols (△,□,○) represent those ones obtained in retrograde perfusion. Data are from 9 (antegrade and retrograde) liver perfusion experiments. Error bars represent mean standard errors.

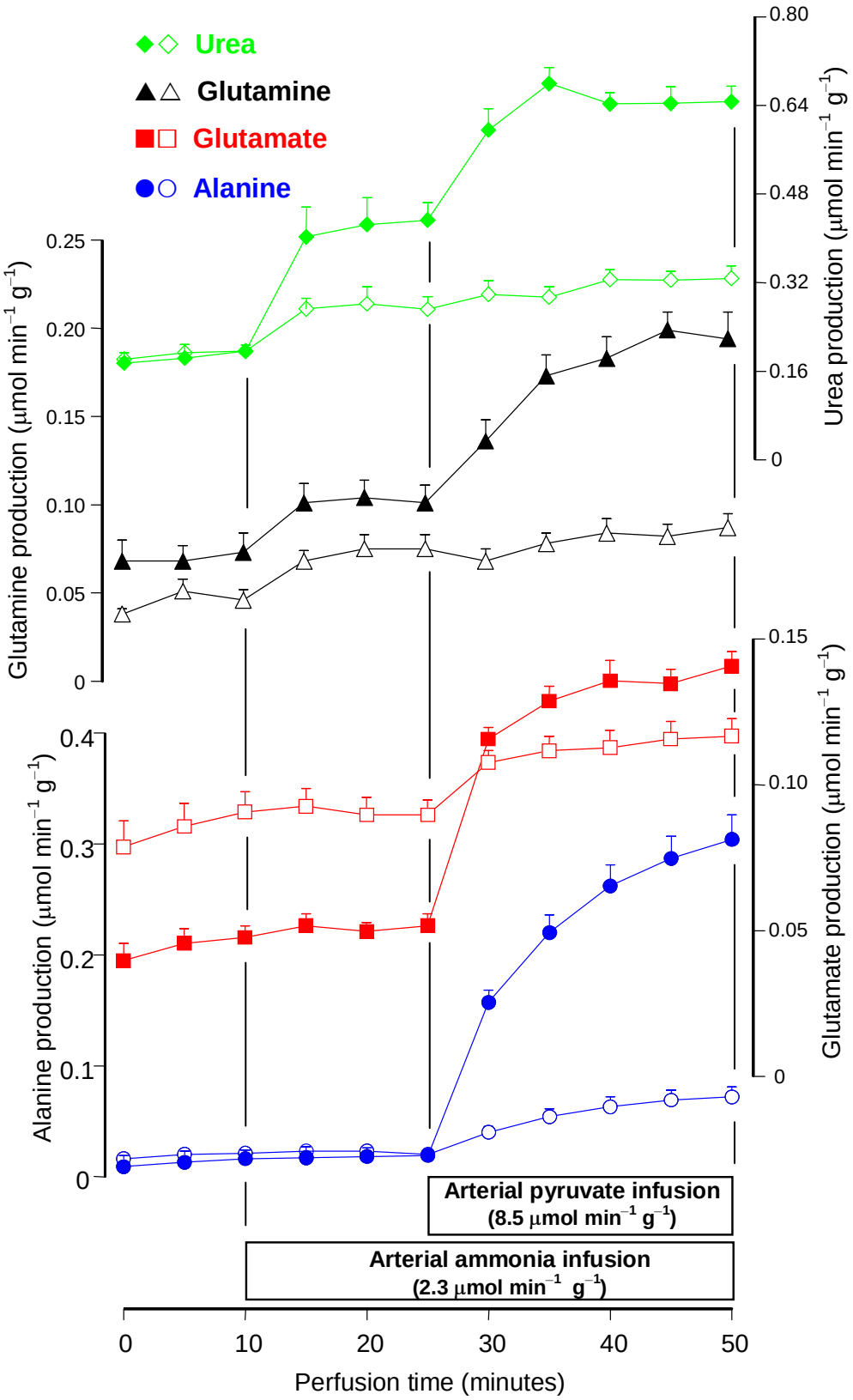

Figure 5. ***The action of arterially infused ammonia and pyruvate on hepatic nitrogen fluxes in antegrade and retrograde perfusion.*** Livers from fasted rats were perfused as described in Materials and Methods. Pyruvate and ammonia were infused into the hepatic artery as indicated. Full symbols (◆,▲,■,●) represent the results obtained in antegrade perfusion; empty symbols (◇,△,□,○) represent those ones obtained in retrograde perfusion. Data are from 9 (antegrade and retrograde) liver perfusion experiments. Error bars represent mean standard errors.

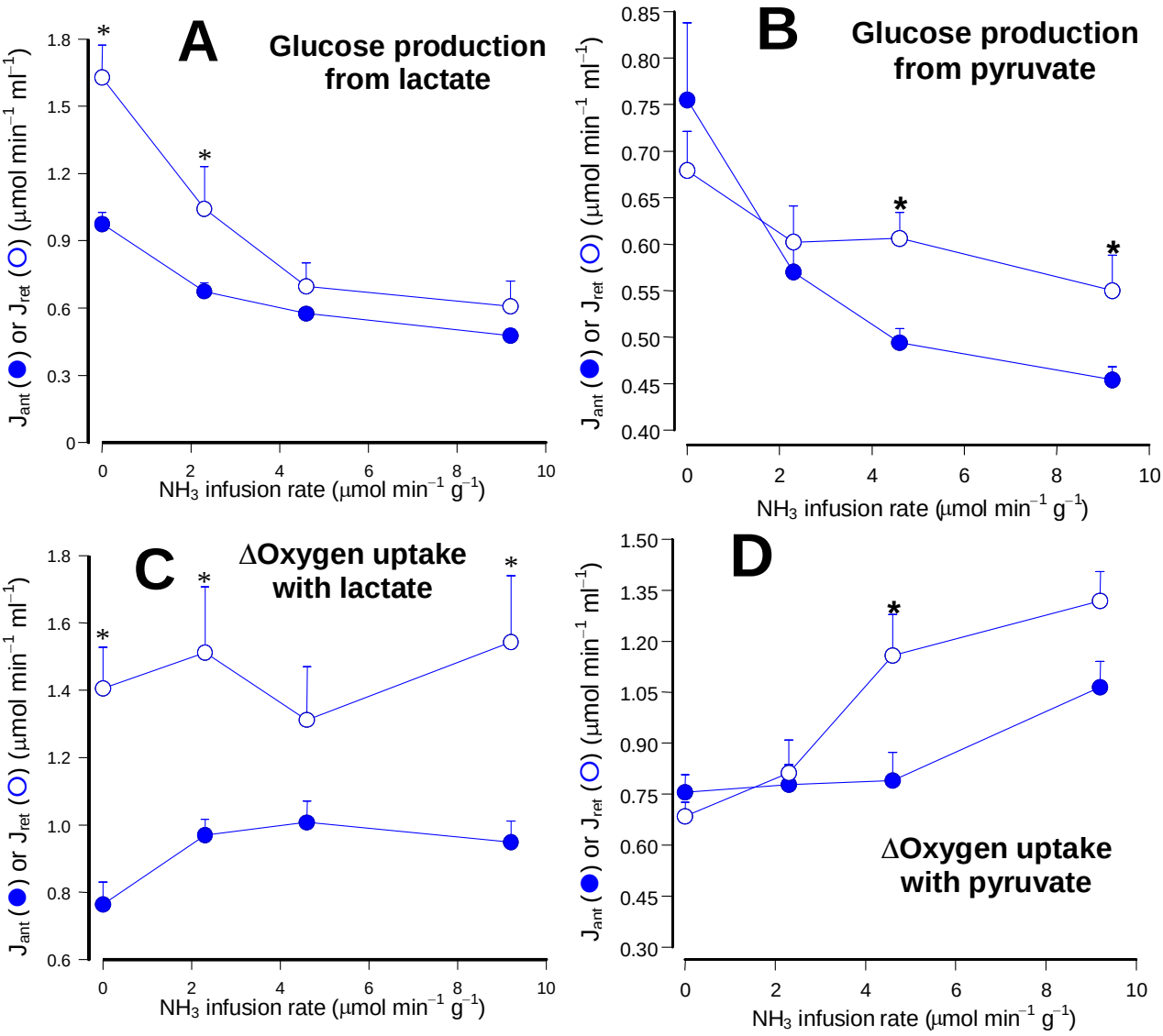

Figure 6. ***Changes in glucose production and oxygen uptake due to lactate + ammonia or pyruvate + ammonia as a function of the arterial ammonia infusion in antegrade (●—●) and retrograde (○—○) perfusion.*** Data from experiments as those ones illustrated by Figures 1 to 4 were used. The changes correspond to the final flux in the presence of both lactate and ammonia (70 minutes perfusion time in Figures 1 and 2) or pyruvate and ammonia (50 minutes perfusion time in Figures 3 and 4) subtracted from the basal flux (no substrates, 0 to 10 minutes perfusion time in Figures 1 to 4). All values were expressed as μmol per minute per ml cell space that is accessible via the hepatic artery in each perfusion mode. These spaces are 0.684 ml/g and 0.266 ml/g in antegrade and retrograde perfusion, respectively (12,13). The data were analyzed by variance analysis; the asterisks indicate significant differences at the 5% level.

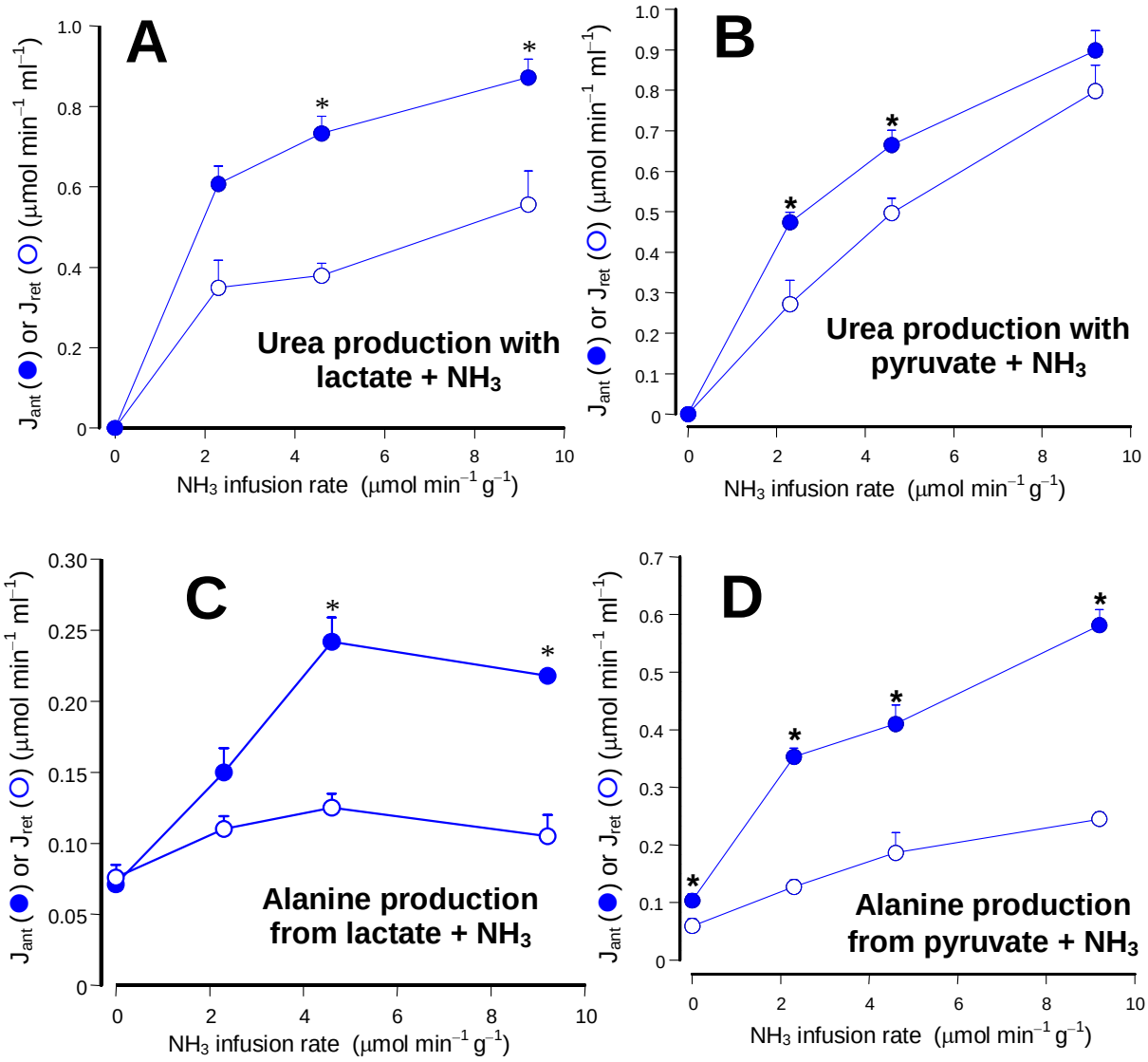

Figure 7. ***Changes in urea and alanine productions due to lactate + ammonia or pyruvate + ammonia as a function of the arterial ammonia infusion in antegrade (●—●) and retrograde (○—○) perfusion.*** Data from experiments as those ones illustrated by Figures 1 to 4 were used. The changes correspond to the final flux in the presence of both lactate and ammonia (70 minutes perfusion time in Figures 1 and 2) or pyruvate and ammonia (50 minutes perfusion time in Figures 3 and 4) subtracted from the basal flux (no substrates, 0 to 10 minutes perfusion time in Figures 1 to 4). All values were expressed as μmol per minute per ml cell space that is accessible via the hepatic artery in each perfusion mode. These spaces are 0.684 ml/g and 0.266 ml/g in antegrade and retrograde perfusion, respectively (12,13). The data were analyzed by variance analysis; the asterisks indicate significant differences at the 5% level.

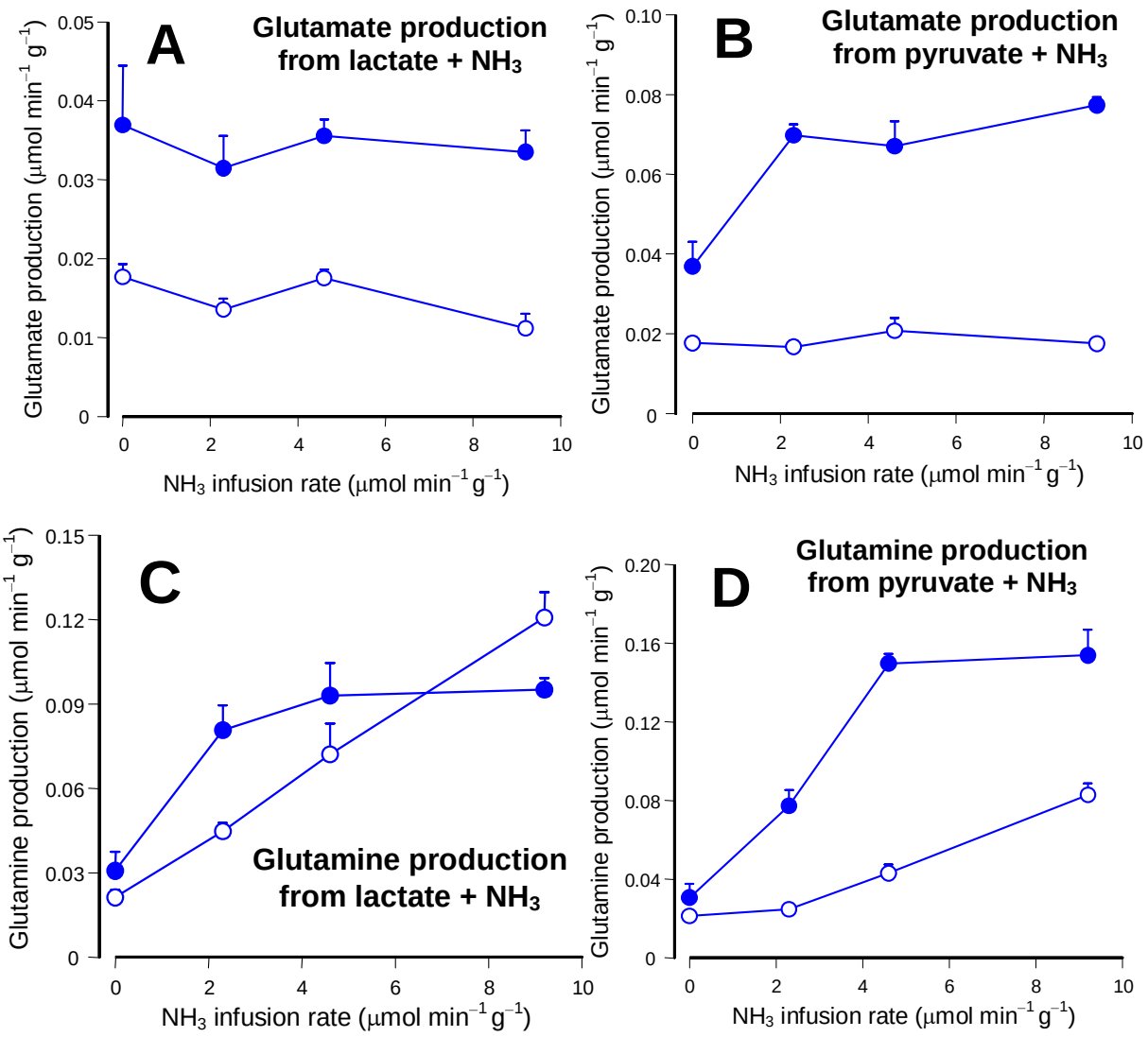

Figure 8. ***Changes in glutamate and glutamine productions due to lactate + ammonia or pyruvate + ammonia as a function of the arterial ammonia infusion in antegrade (●—●) and retrograde (○—○) perfusion.*** Data from experiments as those ones illustrated by Figures 1 to 4 were used. The changes correspond to the final flux in the presence of both lactate and ammonia (70 minutes perfusion time in Figures 1 and 2) or pyruvate and ammonia (50 minutes perfusion time in Figures 3 and 4) subtracted from the basal flux (no substrates, 0 to 10 minutes perfusion time in Figures 1 to 4). All values were expressed as µmol per minute per gram liver wet weight.

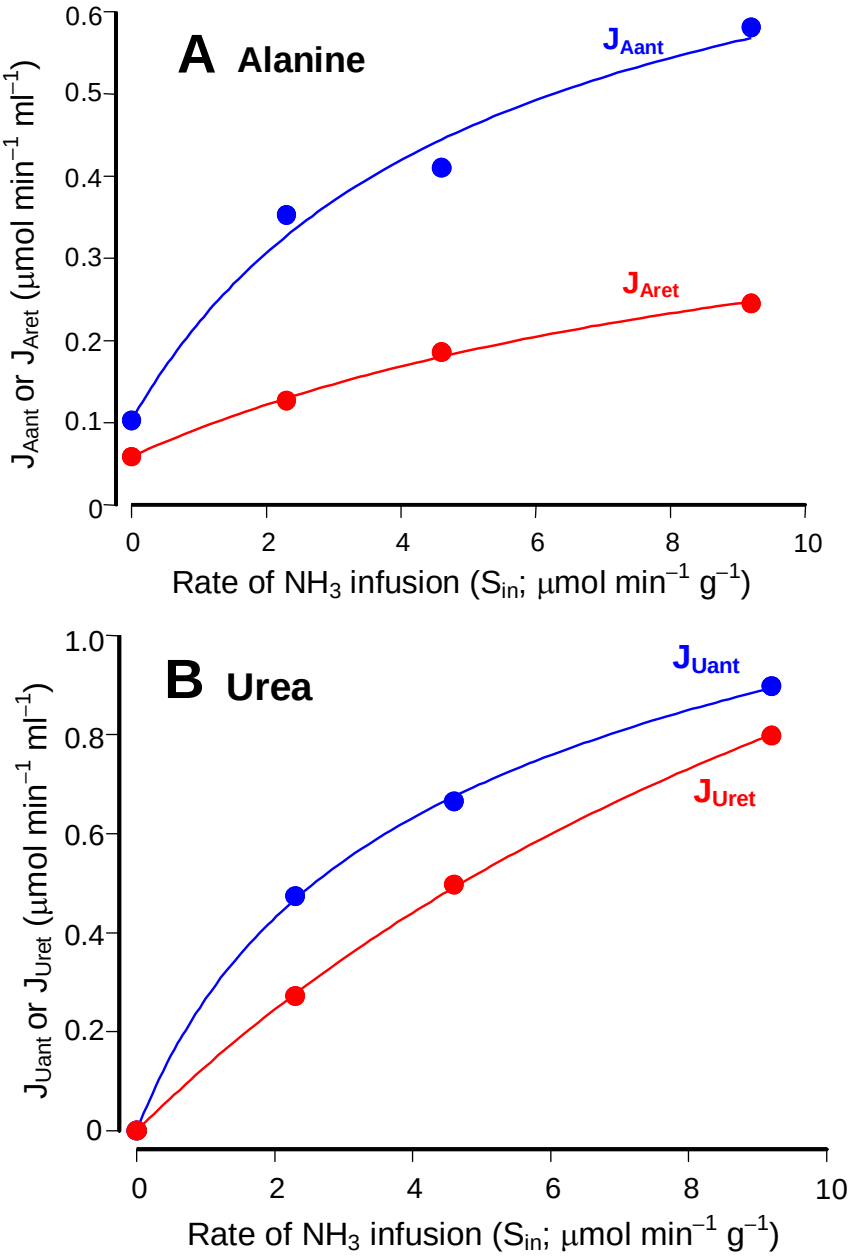

Figure 9. **Experimental and theoretical curves of alanine and urea productions due to pyruvate and ammonia infusion in antegrade (J_{ant}) and retrograde (J_{ret}) perfusion.** Equations [3] and [4] were fitted simultaneously to the experimental urea and alanine productions in antegrade and retrograde perfusion shown in Figures 7B and 7D, respectively. The points represent the experimental values and the continuous lines were calculated according to equations [3] and [4] using the parameters that were optimized by means of a non-linear least-squares procedure (Scientist®). The optimized parameters were the following: F_{AIS} = 0.205 ml min^{-1} g^{-1}; K_{A1} = 1.897 mM; K_{A2} = 1.294 mM; V_{A1} = 0.421 µmol min^{-1} ml^{-1}; V_{A2} = 0.979 µmol min^{-1} ml^{-1}; K_{U1} = 2.606 mM; K_{U2} = 0.754 mM; V_{U1} = 2.154 µmol min^{-1} ml^{-1}; V_{U2} = 1.355 µmol min^{-1} ml^{-1}; The experimentally determined parameters were: F_T = 3.5 ml min^{-1} g^{-1}; F_A = 0.35 ml min^{-1} g^{-1}; r = 0.38. The standard deviation of the estimate was 0.0180 and the coefficient of determination 0.998.

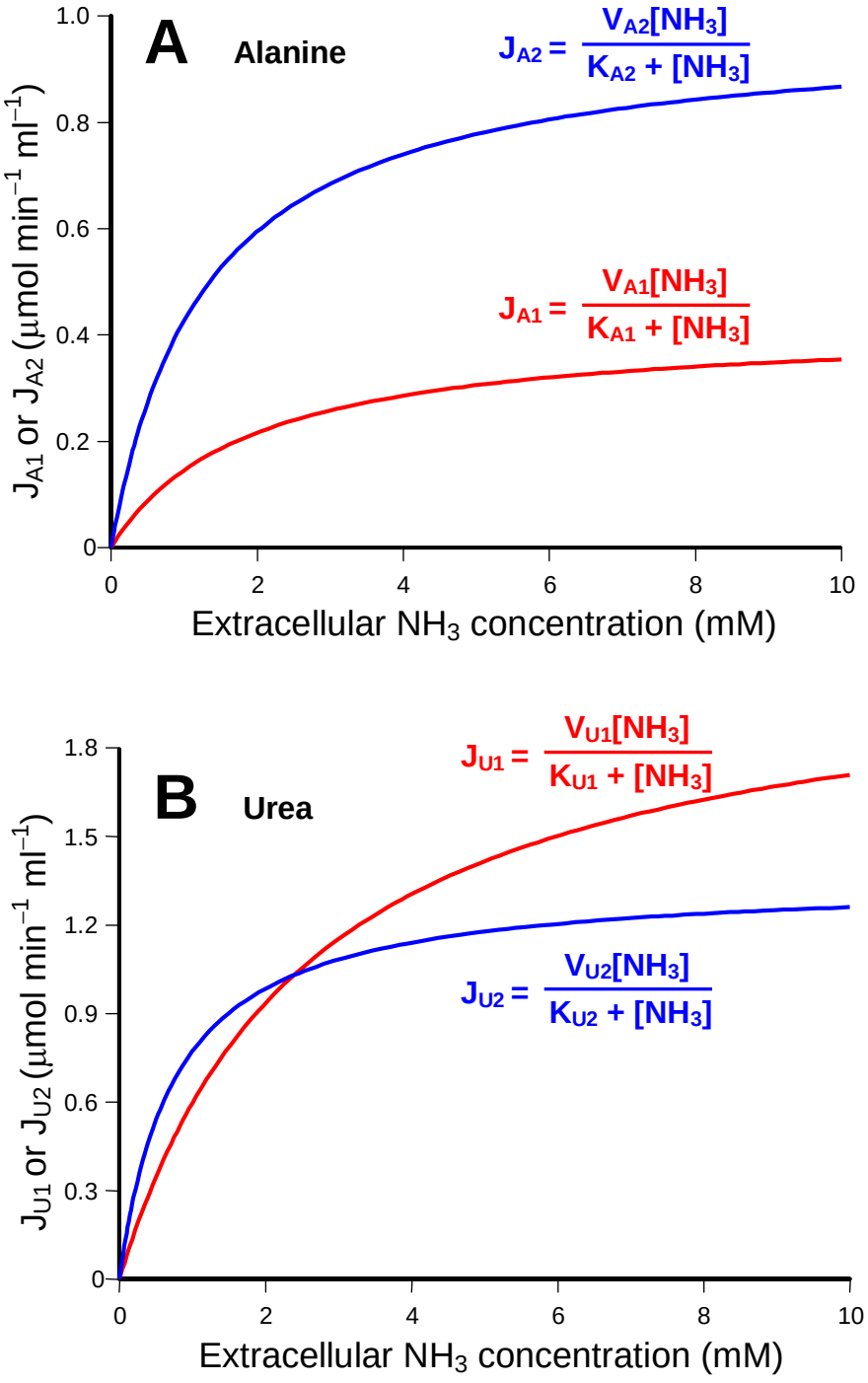

Figure 10. **Expected alanine and urea productions from pyruvate and ammonia in two different zones along the hepatic parenchyma as a function of the extracellular ammonia concentration.** J_{A1} and J_{U1} are the alanine and urea productions in the periportal region situated upstream to the intrasinusoidal confluence, whereas J_{A2} and J_{U2} are the corresponding values in the region situated downstream to the same confluence (see Figure 1). The curves represent Michaelis-Menten functions, calculated using the optimized parameters listed in the legend to Figure 9.

Hepatic zonation of carbon and nitrogen fluxes derived from glutamine and ammonia transformations

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Running tittle: Hepatic zonation of carbon and nitrogen fluxes.

Abstract

The enzyme glutaminase predominates in periportal hepatocytes and it has been proposed it determines the glutamine derived nitrogen flow through the urea cycle. Glutamine derived urea production should, thus, be considerably faster in periportal hepatocytes. This postulate that has not yet been unequivocally demonstrated. Actually, ureogenesis from alanine and from lactate plus ammonia has been found to predominate in perivenous hepatocytes. This makers a direct investigation of ureogenesis from glutamine highly desirable. In the present work this question was investigated in the bivascularly perfused rat liver with [U-^{14}C]glutamine infusion (0.6 mM) into the portal vein (antegrade perfusion) or into the hepatic vein (retrograde perfusion). Glutamine transformation was stimulated by ammonia infusion into the hepatic artery at various concentrations; infusion in retrograde and antegrade perfusion allows to stimulate glutamine metabolism in the periportal region and in the whole liver parenchyma, respectively. The results revealed that the space normalized glutamine uptake, indicated by $^{14}CO_2$ production, predominated in the periportal region. The same was found for gluconeogenesis and the associated oxygen uptake. Ureogenesis, however, was uniformly distributed over the whole liver parenchyma. The current view of the hepatic ammonia-detoxifying system proposes that only a few glutamine synthesizing perivenous cells remove a minor fraction of ammonia that escapes from ureogenesis in periportal cells. However, even when transforming solely glutamine, ureogenesis was more or less uniformly distributed over the whole liver parenchyma. This could be indicating that ureogenesis is an important ammonia-detoxifying mechanism also in cells situated downstream to the periportal region.

Introduction

Glutamine is one of the most abundant amino acids in the organism of mammals and it is involved in more metabolic processes than any other amino acid (Stumvoll *et al*., 1999). Also for the liver the role of glutamine is very important. It is known that the metabolism of glutamine presents zonation (Jungermann & Katz, 1982), i.e., the different regions along the hepatic acini respond in a different way to the amino acid (Häussinger, 1983; Häussinger, 1990; Häussinger *et al*., 1992). Knowledge about zonation of the metabolism of L-glutamine is centered mainly on nitrogen metabolism. The dominant idea is that, along the hepatic acinus, the pathways of urea production and glutamine synthesis are arranged in sequence in order to optimize ammonia detoxification. The urea synthesis in the periportal region represents the system of low affinity for ammonia detoxification. Glutamine synthesis in the perivenous zone represents the system of high affinity for ammonia detoxification. The periportal glutaminase (Watford and Smith, 1990), located in the mitochondria, is stimulated by ammonia and influenced by pH and hormones (Häussinger and Sies, 1979; Squires et al., 1997). The activity of this enzyme is believed to determine, partly at least, the flow of nitrogen derived from glutamine through the urea cycle (Moorman et al., 1994). The glutamine synthase, amply concentrated in a limited number of perivenous hepatocytes, is believed to act as a kind of scavenger for the ammonia that escapes from the periportal urea synthesis.

If the activity of glutaminase determines the nitrogen flow derived from glutamine through the urea cycle, urea production from glutamine should be considerably faster in periportal hepatocytes (Häussinger *et al*., 1992; Moorman et al., 1994). This is a postulate that has not yet been unequivocally demonstrated. Recent studies have shown that urea production from alanine, lactate + ammonia and pyruvate + ammonia is faster in cells situated downstream to the periportal zone at most substrate concentrations (Botini et al., 2005; Comar et al., 2007) in spite of the observation that the expression of key enzymes from the urea cycle predominates in these cells (Braeuning et al., 2006). Periportal predominance of urea production was found only at high pyruvate concentrations, i.e., under highly oxidizing conditions (Comar et al., 2007). Absence of correlation between enzyme activity or enzyme expression and metabolic fluxes in the cell are actually quite common and direct measurements of the latter are indispensable if the behavior of the cell is to be fully understood. Moreover, glutamine is also a gluconeogenic substrate and it has been found that periportal and perivenous cells present

different glucose to urea production ratios from alanine (Botini et al., 2005). This is an important observation if one takes into account the reciprocal regulation of both ureogenesis and gluconeogenesis (Meijer et al., 1978; Martin-Requero et al., 1993), which seems to be different in periportal and perivenous cells, and raises the question about the ureogenesis to glutamine transformation ratios. These and other reasons prompted us to undertake a detailed investigation of the zonation of glutamine transformation with the simultaneous measurement of nitrogen and carbon fluxes. The methodology to be utilized is the bivascularly perfused rat liver, which allows to reach selectively periportal hepatocytes via the hepatic artery in retrograde perfusion (Pang et al. 1988) and which has been successfully used for investigating hepatic zonation without significant alterations of the liver structure (Suzuki-Kemmelmeier et al., 1992; Botini et al., 2005).

Materials and methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the metabolite assays were purchased from Sigma Chemical Co. (St Louis, USA). [U-^{14}C]Glutamine (258 mCi/mol) was purchased from Amersham Bioscience (Buckimghamshire, UK). All standard chemicals were from the best available grade (>99.5 % purity) and were purchased from Merck (Darmstadt, FRG), Carlo Erba (São Paulo, Brasil) and Reagen (Rio de Janeiro, Brazil).

Bivascular liver perfusion

Male albino rats (Wistar), weighing 180-220 g, were fed *ad libitum* with a standard laboratory diet (Purina®). Food was withdrawn 24 hours prior to the liver perfusion experiments. For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). All experiments were done in accordance with the recommendations of the institutional ethics committee for animal experimentation.

Hemoglobin-free, non-recirculating bivascular liver perfusion was performed either in the antegrade mode (entry via the portal vein plus hepatic artery and exit via the hepatic vein) or in the retrograde mode (entry via the hepatic vein plus hepatic artery and exit via the portal vein). The surgical procedure described by Suzuki-Kemmelmeier et al. (1992) was employed. *In situ* perfusion was carried out, the flow being provided by two peristaltic pumps. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine-serum albumin, saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment (37°C). The portal flow was adjusted between 28 and 32 ml/min and the arterial flow between 2 and 3 ml/min. All perfusion experiments were initiated in the antegrade mode. Retrograde perfusion was established by changing the direction of flow at 15-20 minutes before initiating sampling of the effluent perfusate. In all perfusion experiments, livers from fasted rats were used so that glycogenolysis and glycolysis from endogenous sources was minimal (Bazotte et al., 1990).

Analytical

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were measured by means of standard enzymatic procedures: glucose, lactate, urea and ammonia (Bergmeyer, 1974). The oxygen concentration in the outflowing perfusate was monitored continuously, employing a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate (Bracht et al., 2003).

The carbon dioxide production from L-[U-^{14}C]glutamine was measured by trapping $^{14}CO_2$ in phenylethylamine (Scholz et al. 1984). Radioactivity was measured by liquid scintillation spectroscopy. The following scintillation solution was used: toluene/ethanol (2/1) containing 5 g/liter 2,5-diphenyloxazole and 0.15 g/liter 2,2-p-phenyl-ene-bis(5-phenyloxazole).

Treatment of data

Statistical analysis of the data was done by means of the Statistica™ program (Statsoft®, 1998).

Results

Liver responses to glutamine and experimental protocol

Investigation of metabolic zonation using the bivascularly perfused rat liver involves the infusion of substrates into the hepatic artery. Preliminary experiments in which this procedure was adopted with glutamine, however, revealed that this substrate inhibits its own metabolism when it is infused into the hepatic artery. When glutamine was infused at a rate of 24 μmol min^{-1} g^{-1} into the portal vein of livers from 24 hours fasted rats, glucose production reached steady-state rates of 0.45±0.01 μmol min^{-1} g^{-1}; an identical infusion into the hepatic artery in antegrade perfusion produced a glucose production rate of only 0.17±0.03 μmol min^{-1} g^{-1}. For the associated increases in oxygen uptake the corresponding values were 1.00±.084 and 0.15±0.01 μmol min^{-1} g^{-1} and for urea production 1.10±0.09 and 0.48±0.08 μmol min^{-1} g^{-1}. These results are in sharp contrast to the results obtained with other substrates such as alanine and lactate for which equivalent infusions into the hepatic artery in antegrade perfusion and into the portal vein produced equivalent metabolic responses (Bracht et al., 1994; Botini et al., 2005). It should be mentioned that equivalent infusions of substrates into the hepatic artery and portal vein actually means higher concentrations in the arterial bed because the purpose is to supply the sinusoidal bed and the hepatocytes with equivalent concentrations. Possibly, these high concentrations of glutamine in the arterial bed are able to trigger some inhibitory response. The infusion of glutamine into the hepatic artery for studying metabolic zonation must thus be excluded. An alternative to this problem would be to utilize an important characteristic of glutamine metabolism, namely, its dependence on ammonia, as reported by earlier work (Häussinger and Sies, 1979). When infused at low physiological concentrations the transformation of glutamine is signfiicant only if ammonia is simultaneously infused. This characteristic allows to use the experimental protocol illustrated by Figure 1. Glutamine can be infused into the portal vein in antegrade perfusion and into the hepatic vein in retrograde perfusion. The metabolic transformation will be significant only after initiation of ammonia infusion. If the latter is infused into the hepatic artery, the metabolic transformation of glutamine will occur along the entire sinusoidal bed, as illustrated by panel A in Figure 1 (Pang et al. 1988). If the infusion of ammonia into the hepatic artery is done in retrograde perfusion, glutamine metabolism will occur solely in periportal cells, as shown in

panel B. For analysis the resulting metabolic fluxes can be normalized by dividing them through the corresponding accessible cell spaces, as determined by previous work (Fernandes et al., 2002; Fernandes et al., 2003).

Time courses of the reponses to glutamine and ammonia infusions

Figures 2 to 4 illustrate the metabolic responses of the perfused rat liver to [U-^{14}C]glutamine and ammonia infusions. All perfusion experiments were done with livers from 24 hours fasted rats in order to minimize interference by glycogen catabolism. The control experiment in Figure 2 confirms the earlier observations that the infusion of glutamine at physiological concentrations (0.6 mM) does not produce significant increases in glucose, urea and ammonia produtions and oxygen uptake even if the infusion time is as long as 90 minutes (Häussinger and Sies, 1979). Ammonia infusion into the hepatic artery, however, increased glutamine metabolism in both antegrade and retrograde perfusion, as illustrated by Figures 3 and 4. Four parameters were measured: urea, glucose and $^{14}CO_2$ productions and oxygen uptake. Lactate and pyruvate productions, which are significant at high glutamine concentrations (2.5. mM; Corbello-Pereira et al., 2004) were not significantly increased at the low physiological glutamine concentrations employed in the present work. Figure 3 shows the time courses of urea and glucose production of experiments in which the initial glutamine infusion (0.6 mM; 14 minutes) into either the portal vein (antegrade) or hepatic vein (retrograde) was followed by an additional arterial ammonia infusion at a rate of 4.6 μmol min^{-1} g^{-1}. Both glucose and urea productions increased significantly only after initiation of ammonia infusion. At 70 minutes perfusion time new steady-state levels were reached. Notably, glucose production in antegrade perfusion experienced a transient decline just after initiation of ammonia infusion before elevating to a new steady-state. Also notable is the observation that glucose production in retrograde perfusion was higher than that in antegrade perfusion in spite of the fact that a considerably smaller cell space is reached by arterially infused ammonia in retrograde perfusion when compared to antegrade perfusion (Fernandes et al., 2002; Fernandes et al., 2003). The time courses of the changes in oxygen uptake and of $^{14}CO_2$ productions are illustrated by Figure 4. Here again the small increments observed during [U-^{14}C]glutamine infusion are in contrast with the pronounced increases caused by arterially infused ammonia in both antegrade and retrograde perfusion, with a similar tendency of reaching state-state levels at the

end of the experiment. Note that there are relatively small, though significant, $^{14}CO_2$ productions even in the absence of ammonia, a phenomenon that was also observed in earlier work (Häussinger and Sies, 1979). They are equal in antegrade and retrograde perfusion, however, reflecting the fact that glutamine infused into either the portal or hepatic veins has access to the same cells. As expected, basal oxygen uptake rates in antegrade and retrograde perfusion were also nearly the same.

Cell space corrected fluxes of glutamine metabolism

The metabolic fluxes in Figures 3 and 4 were expressed as μmol min^{-1} g^{-1}. This is valid for comparing basal rates or rates before ammonia infusion because glutamine into the portal or hepatic veins has access to the whole liver parenchyma. In order to compare the increments caused by ammonia, however, the metabolic fluxes must be normalized with reference to the cell spaces that are accessible via the hepatic artery in antegrade and retrograde perfusion. These spaces have already been determined by previous work and they are equal to 0.684 and 0.266 ml/g, respectively, for antegrade and retograde perfusion (Fernandes et al., 2002; Fernandes et al., 2003). For the normalization process the rates before ammonia infusion were subtracted from the rates at the end of the ammonia infusion period (70 minutes perfusion time in Figures 3 and 4); this difference was than divided through the corresponding accessible cell space. Implicit in this procedure is the assumption that the increments caused by ammonia reflect solely the contribution of the accessible cell spaces, whereas the basal rates represent the contribution of the whole liver parenchyma (see Figure 1).

In Figure 5 the normalized metabolic changes in antegrade (J_{ant}) and retrograde (J_{ret}) perfusion, calculated as described above, were represented against different infusion rates of ammonia in the range between 1.25 to 9.2 μmol min^{-1} g^{-1}. These infusion rates into the hepatic artery can be expected to generate sinusoidal concentrations between 0.25 and 2.0 mM. It is apparent from the various panels in Figure 5 that the ammonia stimulated glutamine metabolism was faster in retrograde perfusion with the exception of urea production for all ammonia infusion rates. The most accentuated difference was that one found with glucose production, but the difference was also significant for $^{14}CO_2$ production and oxygen uptake. Since only periportal cells are reached by arterially infused ammonia in retrograde perfusion (see Figure 1), this means also a clear periportal predominance for these

parameters. It should be stressed that the $^{14}CO_2$ production has been regarded as an indicator for the total transformation of glutamine, which is thus clearly predominant in the periportal region. On the other hand, the near equality of J_{ant} and J_{ret} for urea production from glutamine reveals absence of periportal predominance for this parameter. This phenomenon, combined with the periportal predominance of glucose production generates unequal ureogenesis/gluconeogenesis ratios along the liver acinus. The mean ureogenesis/gluconeogenesis ratio in antegrade perfusion was equal to 10.7, whereas the corresponding ratio in retrograde perfusion was smaller, namely 3.60. The ratio of ureogenesis to $^{14}CO_2$ production presented a similar relative difference in antegrade and retrograde perfusion, namely 0.89 and 0.49, respectively.

Discussion

The results of the present article confirm a more elevated rate of glutamine uptake in the periportal region. This is indicated by three parameters, $^{14}CO_2$ production from labeled glutamine, gluconeogenesis and the corresponding oxygen uptake increments, which were all more pronounced in retrograde when compared to antegrade perfusion when normalized by the corresponding cell spaces that were accessible to ammonia via the hepatic artery. As shown by previous analysis (Pang et al., 1989; Botini et al., 2005; Comar et al., 2007), when the normalized rates, dependent on the infusion of substrates or effectors into the hepatic artery in retrograde perfusion (J_{ret}) are higher than the corresponding rates measured in antegrade perfusion (J_{ant}), this means always an enrichment of metabolic activity in the periportal space accessible via the intrasinusoidal confluence in comparison to the mean metabolic activity of the liver (see Figure 2). On the other hand, the difference in J_{ret} and J_{ant} for gluconeogenesis was more pronounced than that for $^{14}CO_2$ production. This observation possibly results from the fact that the difference in $^{14}CO_2$ production reflects solely the unequal distribution of the ammonia-dependent glutaminase along the hepatic acinus (Watford and Smith, 1990), whereas the difference in gluconeogenesis reflects both the unequal distributions of glutamine and of several enzymes of the gluconeogenic pathway which also predominate in the periportal region (Jungermann and Katz, 1982).

The J_{ant} and J_{ret} values for ureogenesis from glutamine + ammonia were always approximately the same for the whole range of ammonia infusion. This can be partly due to the lower ammonia concentration in the region between the pre- and intrasinusoidal concentration (see Figure 2). As determined previously, the arterial flow reaching the intrasinusoidal confluences corresponds to 58% of the total arterial flow (Comar et al., 2007). Consequently, the concentration of ammonia in the periportal region during retrograde perfusion will be always 58% of the maximal concentration reached in downstream localized regions (mainly perivenous cells) during antegrade perfusion. This means that the lowest ammonia infusion rate employed in the present study, 1.25 μmol min^{-1} g^{-1}, would have to be increased to 2.15 μmol min^{-1} g^{-1} in retrograde perfusion in order to reach in the periportal region a sinusoidal ammonia concentration comparable to the concentration reached in downstream localized regions in antegrade perfusion (approximately the physiologically concentration of 0.25 mM). Examination of the

tendencies in the response versus infusion rate curves in Figure 5D, however, reveals that one such increase would not produce a significant increment in J_{ret} relative to J_{ant}. It can thus be concluded that, at least for the physiological ammonia concentrations, no periportal predominance of ureogenesis from glutamine + ammonia occurs. This conclusion is fully corroborated by the observation that the mean ureogenesis/gluconeogenesis and ureogenesis/$^{14}CO_2$ production ratios were considerably higher in antegrade perfusion, suggesting that by no means the periportal carbon fluxes derived from glutamine transformation are compatible with the needs in terms of ammonia detoxification which must be more accelerated in downstream localized cells. It should be noted that these results are similar to those found with alanine (Botini et al., 2005) and lactate + ammonia or pyruvate + ammonia (Comar et la., 2007) as substrates for which no periportal predominance of ureogenesis was found.

Periportal predominance of the activity and expression of key enzymes of the urea cycle has been found in several studies (Gaasbeek-Janzen et al., 1984; Braeuning et a., 2006). However, the enzymes of the urea cycle are present in more than 90% of the parenchymal cells. The key-enzyme carbamoyl phosphate synthase, for example, seems to be absent only from those hepatocytes immediately surrounding the hepatic venules (Gaasbeek-Janzen et al., 1984). The hepatocytes not containing carbamoyl phosphate synthase are precisely those ones containing glutamine synthase, which have been estimated as comprising only 7% (Gebhardt et al., 1991). Most hepatocytes, thus, are perfectly able to synthesize urea and the final ureogenic activity will depend not only on the maximal activity (which is that one detected under artificial conditions) but also on the real cellular conditions in terms of the concentrations of substrates and allosteric regulators. In previous experiments, conducted with lactate + ammonia and pyruvate + ammonia as precursors of glucose and urea, clear predominance of urea production in cells localized downstream to the periportal region was found for most conditions (Comar et al., 2007). The exception was the condition pyruvate + high ammonia concentrations, for which periportal predominance of ureogenesis was found. It is important to emphasize the difference between these results (Comar et al., 2007) and the results of the present work. With lactate or pyruvate plus ammonia, ureogenesis is lower in periportal cells when compared to downstream localized ones, whereas with glutamine plus ammonia an almost uniform distribution along the hepatic acinus was found. The presence of glutamine

plus ammonia seems thus to be more favorable for ureogenesis in periportal cells than the condition lactate or pyruvate plus ammonia. Even so, since a clear periportal predominance of ureogenesis was not found in spite of the potentially higher activities of the ureogenic enzymes, it seems likely that some factors must be limiting ureogenesis in the periportal region. With the available data only hypothetical possibilities can be discussed here. The production of N-acetyl-glutamate, the key activator of carbamoyl-phosphate synthase (McGivan et al., 1976) should not be restrained by the periportal availability of glutamate, which is the immediate product of glutamine deamination. It should be remembered, however, that the N-acetyl-glutamate synthase is itself a regulatory enzyme, dependent on regulatory mechanisms with participation of arginine (Kawamoto et al., 1982) and ornithine (Cohen et al., 1980) whose concentrations are not necessarily equal along the hepatic acinus. Aspartate, is an essential amine group donnor for the urea cycle and it is produced by the aspartate transaminase reaction. This enzyme is said to be more active in periportal cells (Agius and Tosh, 1990; Boon et al., 1999), but its activity depends on the availability of oxaloacetate and glutamate. The availability of the latter, as already mentioned, should not be a limting factor. Oxaloacetate, however, could be a limiting factor in periportal cells. This could be happening, for example, when a high gluconeogenic activity combines with a relatively reduced state of the malate dehydrogenase reaction, which is detrrimental to the oxaloacetate concentration.

The current view of the hepatic ammonia-detoxifying system proposes that the small perivenous fraction of glutamine synthesizing perivenous cells removes a minor fraction of ammonia that escapes from ureogenesis in periportal cells (Häussinger, 1983). It certainly continues to be a valid assumption that the perivenous cells immediately surrounding the hepatic venules, which contain glutamine synthase and do not contain carbamoyl phosphate synthase, are able to remove ammonia solely by glutamine synthesis (Gebhardt et al., 1991; Gaasbeek-Janzen et al., 1984). However, ureogenesis can be very active in cells situated downstream to the periportal zone. Under some conditions it can even be more active in these cells than in the periportal cells (Comar et al., 2007). This set of observations indicates that ureogenesis is also an important ammonia-detoxifying mechanism in the perivenous region excepting only the small fraction of cells deprived from carbamoyl-phosphate synthase.

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Figure 1

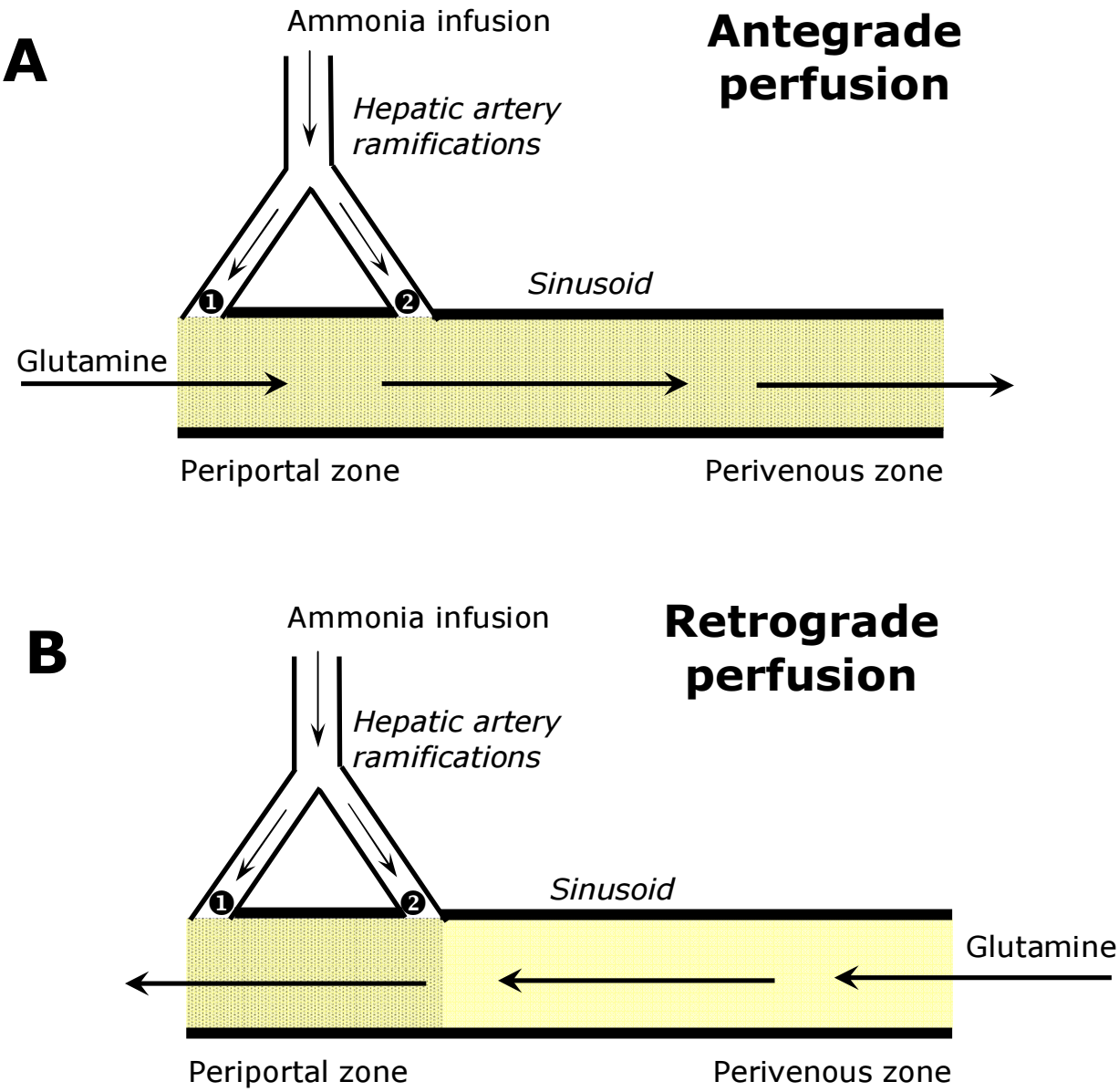

Figure 1. ***Schematic representation of some characteristics of the hepatic microcirculation and the experimental protocols.*** The arrows indicate the direction of flow. Legends: ❶, the presinusoidal confluence of the arterial and portal bed; ❷, the intrasinusoidal confluence of the arterial and portal bed.

Figure 2

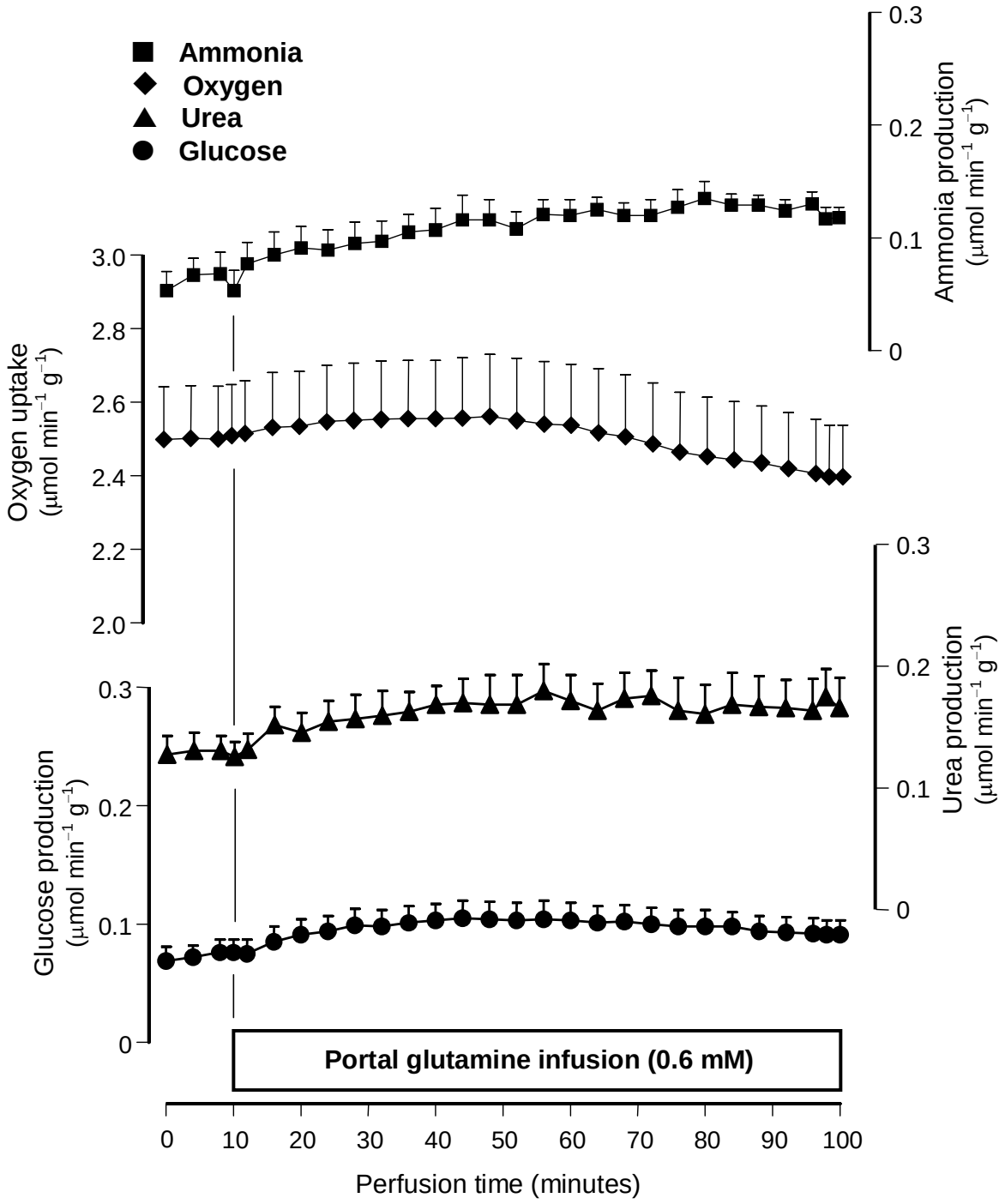

Figure 2. ***Time courses of ammonia, urea and glucose productions and oxygen uptake during portal infusion of 0.6 mM glutamine.*** Livers from fasted rats were perfused as described in Materials and Methods. Glutamine was infused as indicated. Data are from 4 liver perfusion experiments and error bars are mean standard errors.

Figure 3

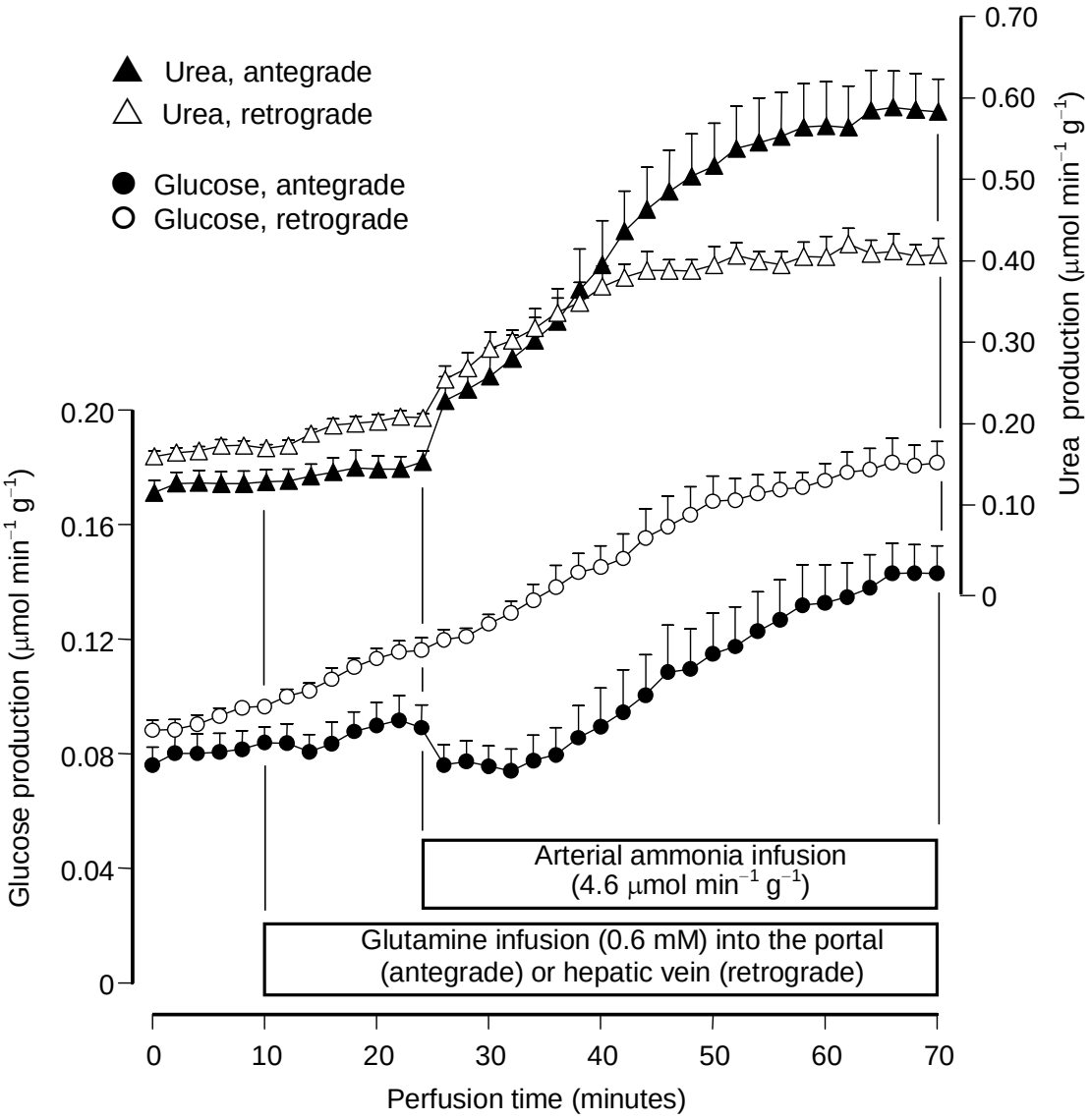

Figure 4. ***Time courses of the actions of arterially infused ammonia on*** **urea and glucose** ***productions from [U-^{14}C]glutamine in antegrade and retrograde perfusion.*** Livers from fasted rats were perfused as described in Materials and Methods. [U-^{14}C]Glutamine and ammonia were infused as indicated. Data are from 5 liver perfusion experiments and error bars are mean standard errors.

Figure 4

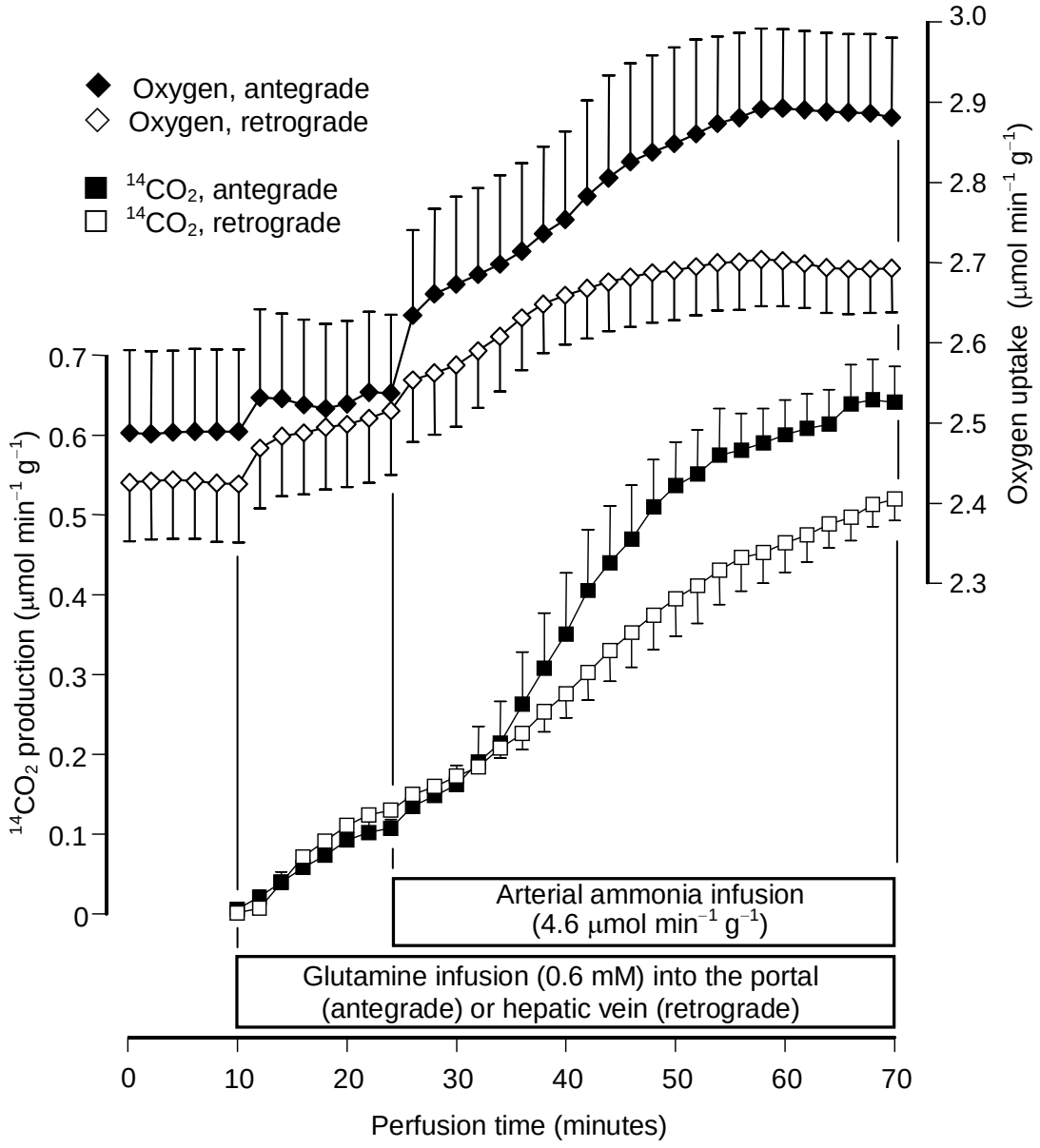

Figure 4. ***Time courses of the actions of arterially infused ammonia on $^{14}CO_2$ production from [U-^{14}C]glutamine and the corresponding oxygen uptake increments in antegrade and retrograde perfusion.*** Livers from fasted rats were perfused as described in Materials and Methods. [U-^{14}C]Glutamine and ammonia were infused as indicated. Data are from 5 liver perfusion experiments and error bars are mean standard errors.

Figure 5

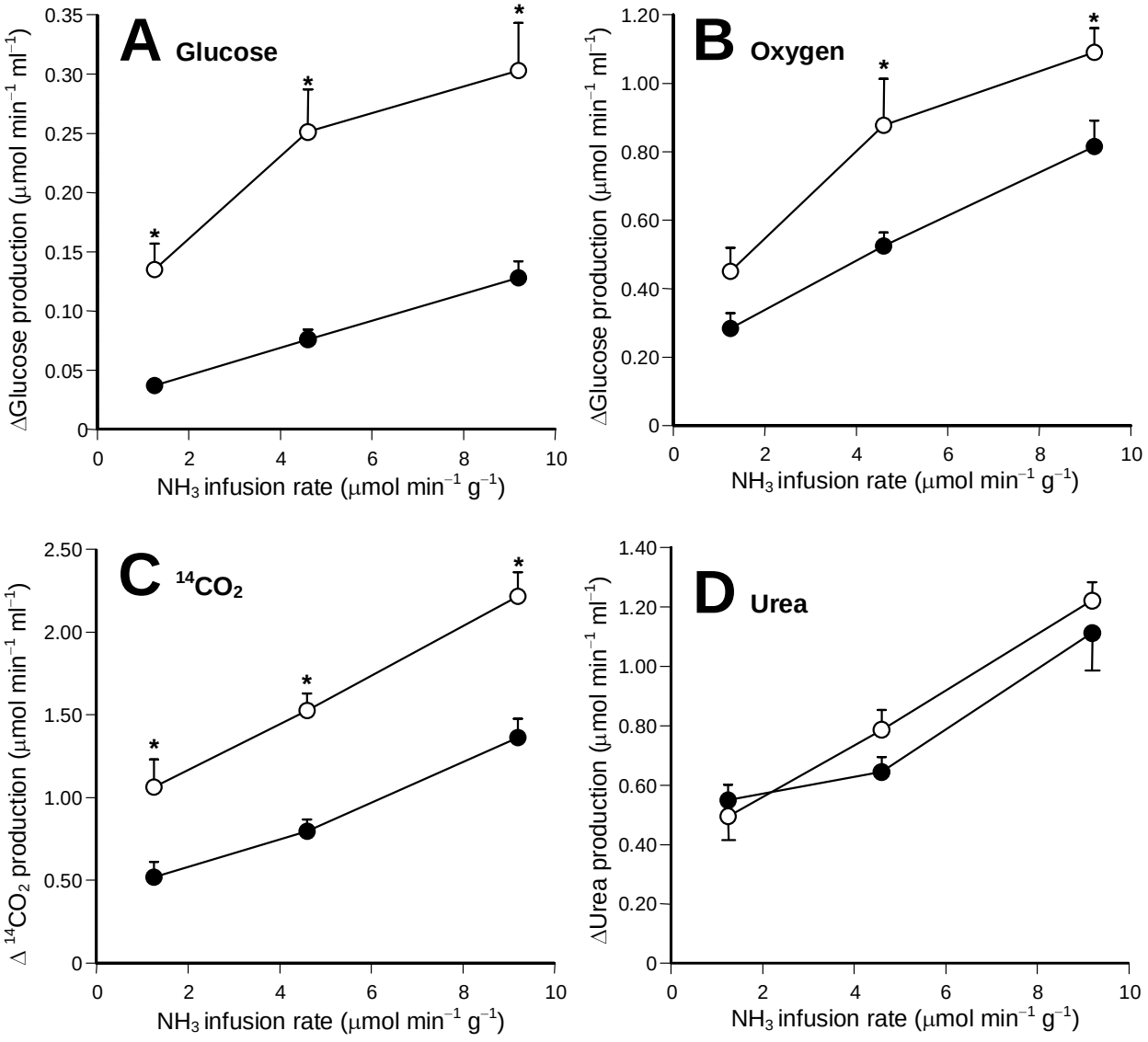

Figure 5. ***Changes in glucose, $^{14}CO_2$ and urea productions and oxygen uptake due to glutamine + ammonia as a function of the arterial ammonia infusion in antegrade (●—●) and retrograde (○—○) perfusion.*** Data from experiments as those ones illustrated by Figures 3 and 4 were used. The glutamine concentration was the same for all experiments, namely 0.6 mM. The changes correspond to the final flux in the presence of both glutamine and ammonia (70 minutes perfusion time in Figures 3 and 4), subtracted from the basal flux in sole presence of glutamine. All values were expressed as μmol per minute per ml cell space that is accessible via the hepatic artery in each perfusion mode. These spaces are 0.684 ml/g and 0.266 ml/g in antegrade and retrograde perfusion, respectively (Fernandes et al., 2002; Fernandes et al., 2003). The data were analyzed by variance analysis; the asterisks indicate significant differences at the 5% level.

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