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Ação do n-propil galato sobre o metabolismo do fígado de rato

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Biografia

Gabrielle Jacklin Eler nasceu em Umuarama, Estado do Paraná no dia 30 de março de 1984. Possui graduação em Enfermagem pela Universidade Paranaense (2006). Durante o período da graduação participou como aluna de iniciação científica do projeto "Efeito da desnutrição protéica sobre o plexo mientérico de ratos adultos", sob orientação da professora Débora de Mello Gonçales Sant'Ana e o professor Eduardo José de Almeida Araújo. Atualmente cursa Mestrado em Ciências Biológicas com área de concentração em Biologia Celular e Molecular, tendo atuado na área de metabolismo hepático, com participação em vários projetos.

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Apresentação

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

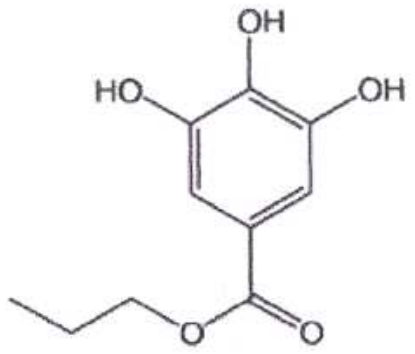
Eler GJ, Peralta RM, Bracht A. The action of n-propyl gallate on gluconeogenesis and oxygen uptake in the rat liver. *Chemico-Biological Interactions* (submetido).

Eler GJ, Peralta RM, Bracht A. The action of n-propyl gallate on glucose output and related parameters under glycogenolytic conditions in the rat liver (a ser submetido).

Os dois artigos relatam e discutem observações originais.

Resumo geral

INTRODUÇÃO E OBJETIVOS — O **n-propil-galato,** ou propil 3,4,5-trihidroxi-benzoato (ver figura), é um éster formado pela condensação do ácido gálico com o n-propanol. O n-propil galato tem sido usado em alimentos (principalmente óleos e gorduras), cosméticos, produtos para cabelos, adesivos e lubrificantes para impedir a oxidação. O composto protege contra a oxidação por peróxido de hidrogênio e radicais livres de oxigênio através de um mecanismo catalítico semelhante ao da superóxido dismutase.



Estrutura do n-propil galato (massa molecular = 212,2 g/mol).

O n-propil galato é encontrado naturalmente em muitos produtos de origem vegetal. No chá verde, por exemplo, a porção ácido gálico é uma das estruturas mais comuns entre os seus compostos fenólicos. O ácido gálico livre e o n-propil galato são particularmente abundantes, mas outros ésteres também são encontrados. O n-propil galato apresenta certo grau de toxicidade, inclusive hepatotoxicidade conforme sugerido por aumentos nas transaminases circulantes. Em hepatócitos foram observados morte celular aguda, inibição do consumo de oxigênio e depleção de ATP. Por isto a ação tóxica do n-propil galato tem sido atribuída a uma ação sobre o metabolismo energético mitocondrial, mas a conclusão baseia-se em experimentos realizados com concentrações bastante altas do composto (1 a 2 mM). No entanto, foi relatado recentemente que o n-propil galato e o ácido gálico agem como eliminadores de radicais livres em concentrações de até 200 μM. Além disto, o ácido gálico, inibe a comunicação intercelular mediada por *gap junctions* nesta mesma faixa de concentração. Deve-se acrescentar ainda que a hidrólise do n-propil galato gera pelo menos um metabólito ativo que é o n-propanol, cujo metabolismo é semelhante ao do etanol, um inibidor da gliconeogênese em consequência de suas reações de transformação. Estes fatos levaram-nos a investigar possíveis ações metabólicas do n-propil galato em concentrações bem abaixo de 1-2 mM. Este objetivo pode ser atingido mais facilmente através de medidas de vias metabólicas biossintéticas, tais como a gliconeogênese, muito sensíveis a alterações na integridade celular ou nas concentrações intracelulares de intermediários-chave do metabolismo. Para medir a gliconeogênese e vias associadas o fígado de rato em perfusão isolada foi utilizado. Adicionalmente, frações sub-celulares, tais como microssomos, foram usados para medidas complementares de atividades enzimáticas com vistas à dedução de mecanismos para os efeitos observados.

MÉTODOS — Ratos Wistar machos (180-220 g), alimentados com ração padronizada (Nuvilab®), foram utilizados. O fígado foi perfundido isoladamente 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. Quatro substratos gliconeogênicos foram infundidos: lactato, glicerol, alanina e frutose. A concentração de oxigênio no perfusado efluente foi monitorada continuamente por polarografia; a produção de metabólitos foi medida enzimaticamente usando métodos espectrofotométricos. Fluxos metabólicos foram calculados a partir

de diferenças porto-venosas e do fluxo total pelo fígado e referidos ao peso fresco do órgão. O consumo de oxigênio por mitocôndrias e microssomos isolados foi medido polarograficamente. Atividades enzimáticas foram medidas espectrofotometricamente e através da incorporação de ^{14}C a partir de [^{14}C]-bicarbonato (reação de carboxilação). A radioatividade foi medida através de espectrometria de cintilação líquida.

RESULTADOS — Sob condições de gliconeogênese (fígados de ratos em jejum de 24 horas), o n-propil galato, na faixa de concentração até 200 μM, inibiu a gliconeogênese com os quatro substratos testados na presente investigação: lactato, alanina, glicerol e frutose. A inibição foi reversível. Com lactato, 50% de inibição foram encontrados na concentração de 86,4 μM. Com 200 μM a inibição subiu para 80% com lactato, mas foi de 99% com alanina. Com frutose e glicerol a inibição foi menos pronunciada, 30 e 27%, respectivamente, com n-propil galato 200 μM. O efeito do ácido gálico sobre a gliconeogênese foi bem menor: uma concentração de 100 μM produziu 20% de inibição da gliconeogênese a partir de lactato. Paralelamente à inibição da gliconeogênese o n-propil galato também aumentou o consumo de oxigênio de modo saturável com 50% de estímulo máximo na concentração de 42,8 μM quando lactato foi o substrato. Este estímulo máximo foi de 1,3 vezes em relação ao estímulo causado pela infusão de lactato. Em termos relativos o estímulo foi maior quando glicerol foi o substrato: 9,5 vezes em relação ao estímulo produzido apenas pelo substrato. A produção de piruvato com todos os substratos exceto a frutose foi estimulada pelo n-propil galato. A produção de lactato também foi estimulada quando alanina e frutose foram os substratos. O ácido gálico, por outro lado, não causou estímulo significativo no consumo de oxigênio.

A atividade do ciclo dos ácidos tricarboxílicos foi medida como produção de $^{14}CO_2$ após marcação dos estoques de acetil-CoA com infusão de quantidades-traço de [1-^{14}C]octanoato. O n-propil galato inibiu reversivelmente a produção de $^{14}CO_2$; esta inibição foi simultânea ao estímulo do consumo de oxigênio.

Ao bloquear a cadeia respiratória mitocondrial com cianeto 2 mM a capacidade do n-propil galato em aumentar o consumo de oxigênio no fígado em perfusão com meio livre sem substratos foi reduzida em 63%. Comparativamente, a mesma concentração de cianeto eliminou por completo as capacidades de aumentar o consumo de oxigênio do n-propanol (200 μM) e do 2,4-dinitrofenol (100 μM). Eliminação total da capacidade do n-propil galato em estimular o consumo de oxigênio foi obtida, no entanto, pela infusão simultânea de cianeto 2 mM e proadifen 100 μM, este último um inibidor da cadeia microssomal de transporte de elétrons.

A liberação de glicose e lactato sob condições glicogenolíticas no figado de ratos alimentados foi aumentada pelo n-propil galato. Os dois efeitos foram relativamente modestos e às vezes instáveis. Na passagem de 200 para 400 μM o efeito sobre a liberação de glicose foi aumentado. O n-propanol, na concentração de 200 μM, também causou pequeno aumento na liberação de glicose.

As velocidades de liberação de n-propanol no perfusado efluente com n-propil galato portal 200, 500 e 1000 μM foram, respectivamente, 0, 0,14±0,02 e 0,61±0,16 μmol min^{-1} g^{-1}. n-Propanol na concentração de 200 μM inibiu claramente a gliconeogênese a partir de lactato de 0,81±0,05 para 0,64±0,03 μmol min^{-1} g^{-1} (21%) sem alterar, neste caso, o consumo de oxigênio.

O n-propil galato até 200 μM não afetou a respiração de mitocôndrias isoladas dependente de succinato e α-cetoglutarato, tanto na ausência como na presença de ADP. Confirmando observações prévias, no entanto, o controle respi-

ratório, foi claramente prejudicado pelo n-propil galato com ambos os substratos. O consumo de oxigênio de microssomos incubados na presença de NADPH, no entanto, foi estimulado de modo saturável pelo n-propil galato (50% do estímulo máximo na concentração de 37,5 μM). O ácido gálico, porém, não causou nenhum estímulo.

Os níveis teciduais de AMP e ADP não foram alterados pelo n-propil galato. Houve, no entanto, uma pequena, mas estatisticamente significativa, redução dos níveis teciduais de ATP (5%).

A atividade da piruvato carboxilase de mitocôndrias intatas foi inibida pelo n-propil galato, com 50% de inibição numa concentração de 142,2 μM. Em mitocôndrias rompidas por congelamento-descongelamento a inibição foi mínima. O ácido gálico não inibiu a atividade piruvato carboxilase. A glicose 6-fosfatase e a frutose 1,6-bisfosfatase não foram inibidas pelo n-propil galato.

DISCUSSÃO E CONCLUSÕES — A mais importante conclusão é que o n-propil-galato é capaz de afetar o metabolismo do fígado de rato sem que haja depleção do ATP intracelular e em concentrações consideravelmente menores do que aquelas que foram relatadas como sendo inibidoras do metabolismo energético mitocondrial em hepatócitos isolados. O efeito mais importante é a inibição da liberação de glicose, mas o composto produz outras alterações em fluxos metabólicos. Entre elas está o estímulo do consumo de oxigênio que ocorre tanto nas mitocôndrias como também no sistema microssomal de transporte de elétrons. A causa mais importante para a diminuição da liberação de glicose parece ser a inibição da gliconeogênese em consequência da inibição da carboxilação do piruvato. Isto, por sua vez, deve ser consequência de uma inibição do transporte de piruvato através da membrana mitocondrial, conforme revelado pela observação de que a inibição da carboxilação ocorre quase que apenas em mitocôndrias intactas. Este não é o único mecanismo de inibição da liberação de glicose, como pode ser deduzido do fato de que o n-propil galato também inibiu, embora com menor intensidade, a liberação de glicose dependente de precursores (frutose e glicerol) cuja entrada na via gliconeogênica está situada após a reação da piruvato carboxilase. Mecanismos adicionais para a inibição da liberação de glicose são: a) desvio de uma fração da glicose 6-fosfato para a via das pentoses com vistas à produção de equivalentes redutores na forma de NADPH, a serem usados no transporte microssomal de elétrons ativado pelo n-propil galato; b) desvio de uma fração da glicose 6-fosfato para as reações de glicuronidação do ácido gálico; c) inibição da gliconeogênese pelo desvio do malato citosólico (em quase-equilíbrio com o oxaloacetato) para a geração de NADPH com vistas à respiração microssomal; d) inibição da gliconeogênese pelo n-propanol, produzido intracelularmente a partir do n-propil galato. A oxidação do n-propanol é provavelmente a principal responsável pelo aumento da respiração mitocondrial causada pelo n-propil galato, a qual é simultânea à ação inibitória sobre o ciclo dos ácidos tricarboxílicos.

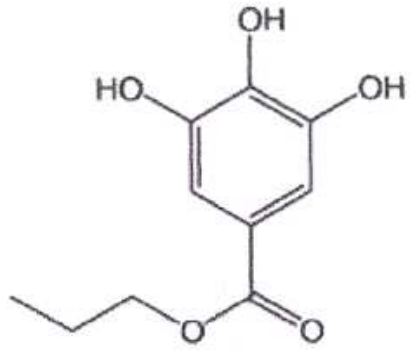
O desacoplamento da cadeia respiratória mitocondrial não parece ser significativo no fígado em perfusão em concentrações de n-propil galato até 200 μM, embora ele seja significativo em incubações com mitocôndrias isoladas. No entanto, concentrações maiores, 400 μM por exemplo, provavelmente produzem desacoplamento em níveis significativos.

Como conclusão final pode-se dizer que o n-propil galato é uma espécie de efetor metabólico, cujas ações sobre o metabolismo hepático são relativamente moderadas embora possam se tornar altamente prejudiciais para o órgão e para o organismo como um todo em altas doses e concentrações.

General abstract

INTRODUCTION AND AIMS — **n-Propyl gallate**, or propyl 3,4,5-trihidroxy-benzoate (see figure), is an ester formed by condensation of gallic acid and n-propanol. It has been added to foods (especially oils and fats), cosmetics, hair products, adhesives and lubricants to prevent oxidation. The compound protects against oxidation by hydrogen peroxide and oxygen free-radicals by means of a catalytic mechanism similar to that of the superoxide dismutase.

n-Propyl gallate occurs naturally in many products of plant origin. In green tea, for example, the gallic acid moiety is one of the most common structures among its phenolic compounds. Free gallic acid and n-propyl gallate are particularly abundant but other esters have also been found. In mammals the n-propyl gallate presents a certain degree of toxicity including hepatotoxicity as suggested by increases in the circulating levels of amino-transferases. Acute cell death, inhibition of oxygen consumption and ATP depletion have been observed in isolated hepatocytes. For this reason the effects of n-propyl gallate have been atributted to an action on the mi-tochondrial energy metabolism, but this con-clusion is based on experiments in which high concentrations of the compound were used (1 a 2 mM). Nevertheless, it has been recently reported that n-propyl gallate and gallic acid act as free-radical scavengers at concentrations up to 200 μM. Furthermore, gallic acid inhibits gap-junctional intercellular communication in the same concentration range. It must also be added that hepatic transformation of n-propyl gallate generates n-propanol, a compound whose metabolic transformation is similar to that of ethanol, an inhibitor of hepatic gluconeogenesis in consequence of its transformation reactions. All these facts prompted us to investigate possible metabolic actions of n-propyl gallate at concen-trations well below 1-2 mM. This purpose can be best accomplished by measuring metabolic biosynthetic pathways, such as gluconeogenesis, that are highly sensitive to changes in cell integrity or to relatively small changes in the intracellular concentration of key metabolic intermediates. For measuring gluco-neogenesis and associated pathways the isolated perfused rat liver was used. Additionally, sub-cellular fractions, such as microsomes, were used for comple-mentary measurements of several enzymatic activities. The latter should allow to suggest possible mechanisms for the observed effects.



Structure of n-propyl gallate (molecular mass = 212.2 g/mol).

METHODS — Male Wistar rats (180-220 g), fed *ad libitum* with a standard laboratory diet (Nuvilab®), were used. The isolated liver was perfused in the non-recirculating mode. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with O_2 and CO_2 (95:5) by means of a membrane oxygenator and simultaneously heated to 37 °C. Four gluconeogenic substrates were infused: lac-tate, glycerol, alanine and fructose. The oxygen concentration in the perfusate was monitored continuously by means of polarography. Metabolite production was measured by means of enzymatic assays. Metabolic fluxes were calculated from the portal-venous differences and the total flow through the liver and referred to the wet liver weight. Oxygen consumption by isolated mitochondria and microsomes

was measured polarographically. Enzyme activities were measured spectrophotometrically or by assaying ^{14}C incorporation from [^{14}C]bicarbonate (carboxylation reaction). Radioactivity was measured by liquid scintillation spectrometry.

RESULTS — Under gluconeogenic conditions (livers of 24-hours fasted rats), n-propyl gallate in the concentration range up to 200 μM, inhibited glucose synthesis from the four substrates tested in the present work: lactate, alanine, glycerol and fructose. The inhibition was reversible. With lactate 50% inhibition was found for a concentration of 86.4 μM. With 200 μM the inhibition increased to 80% with lactate, but it was 99% with alanine. With the substrates fructose and glycerol the inhibition was less pronounced, 30 and 27%, respectively, with 200 μM n-propyl gallate. The action of gallic acid on gluconeogenesis was considerably smaller: the concentration of 100 μM produced 20% inhibition when lactate was the substrate. In parallel with the inhibition of gluconeogenesis, n-propyl gallate also increased oxygen uptake in a saturable manner with 50% of maximal stimulation at the concentration of 42.8 μM when lactate was the substrate. The maximal stimulation corresponded to 1.3 times the stimulation caused by lactate infusion alone. The relative stimulation was much more pronounced when glycerol was the substrate: 9.5 fold in relation to the stimulation caused by the substrate alone. Pyruvate productions from all substrates except fructose were stimulated by n-propyl gallate. Lactate productions from alanine and fructose were also stimulated. Gallic acid, on the other hand, did not produce significant stimulation of oxygen uptake.

The activity of the tricarboxylic acid cycle was quantified as $^{14}CO_2$ production after labelling the cellular acetyl-CoA pools with the infusion of tracer amounts of [1-^{14}C]octanoate. n-Propyl gallate reversibly inhibited $^{14}CO_2$ production; this inhibition occurred simultaneously with a stimulation of oxygen uptake.

Blocking of the mitochondrial respiratory chain of livers perfused with substrate-free perfusion fluid with 2 mM cyanide diminished the n-propyl gallate stimulation of oxygen uptake by 63%. Comparatively, the same cyanide concentration abolished the stimulating action of n-propanol (200 μM) and 2,4-dinitrophenol (100 μM). Total impairment of the capacity of n-propyl gallate as an oxygen uptake stimulator was obtained, however, by the simultaneous infusion of 2 mM cyanide and 100 μM proadifen, the latter being an inhibitor of the microsomal electron transport chain.

Glucose and lactate output under glycogenolytic conditions in livers from fed rats were increased by n-propyl gallate. Both effects were relatively mild and unstable under some conditions. In the passage from 200 μM to 400 μM n-propyl gallate the action on glucose release was further enhanced. n-Propanol at the concentration of 200 μM also produced small increases in glucose release.

The rates of n-propanol overflow in the effluent perfusate with portal n-propyl gallate 200, 500 and 1000 μM were, respectively, 0, 0.14±0.02 and 0.61±0.16 μmol min^{-1} g^{-1}. n-Propanol at the concentration of 200 μM clearly inhibited lactate gluconeogenesis from 0.808±0.051 to 0.639±0.031 μmol min^{-1} g^{-1} (21%) without changing, under these conditions, oxygen uptake.

n-Propyl gallate up to 200 μM did not increase respiration driven by succinate and α-ketoglutarate in intact mitochondria either in the absence or presence of exogenously added ADP. Confirming previous reports, however, the respiratory control, was clearly impaired by n-propyl gallate with both substrates. Oxygen uptake of microsomes incubated in the presence of NADPH was stimulated in a saturable manner by n-propyl gallate (50% of maximal stimulation at 37.5 μM).

Gallic acid, however, did not stimulate NADPH driven microsomal oxygen uptake.

The tissue levels of AMP and ADP were not affected by n-propyl gallate up to 200 μM. There was, however, a small, though statistically significant, reduction in the tissue levels of ATP (5%).

The activity of pyruvate carboxylase of intact mitochondria was inhibited by n-propyl gallate with 50% inhibition at the concentration of 142.2 μM. There was only minimal inhibition of the pyruvate carboxylase activity in freeze-thawing disrupted mitochondria. Gallic acid did not inhibit the pyruvate carboxylase activity of intact or disrupted mitochondria. Glucose 6-phosphatase and fructose 1,6-bisphosphatase activites were not inhibited by n-propyl gallate.

DISCUSSION AND CONCLUSIONS — The most important conclusion is that n-propyl gallate is able to affect the metabolism of the rat liver without cellular ATP depletion at concentrations considerably smaller than those reported to be inhibitory for the mitochondrial energy metabolism in isolated hepatocytes. The most prominent effect is inhibition of glucose output, but the compound is able to produce several alterations in the hepatocyte in terms of metabolic fluxes. One of these alterations is oxygen uptake stimulation which occurs in both mitochondria and microsomes. The most important cause for the diminution of glucose output in the absence of significant glycogen stores seems to be inhibition of gluconeogenesis in consequence of an inhibition of pyruvate carboxylation. This, in turn, results from an inhibition of pyruvate transport across the mitochondrial membrane, as revealed by the observation that inhibition of carboxylation occurs only in intact mitochondria. This is not the only mechanism, as can be deduced from the fact that n-propyl gallate also inhibits, though less strongly, glucose synthesis from precursors (fructose and glycerol) whose entry into the gluconeogenic pathway is situated after the pyruvate carboxylase reaction. Additional mechanisms for glucose output inhibition are: a) deviation of a fraction of glucose 6-phosphate into the pentose monophosphate shunt for the production of reducing equivalents in the form of NADPH to be used in the n-propyl gallate-stimulated microsomal electron transport; b) deviation of a fraction of glucose 6-phosphate for glucuronidation reactions; c) inhibition of gluconeogenesis by deviating cytosolic malate (in equilibrium with oxaloacetate) for the production of NADPH for microsomal electron transport; d) gluconeogenesis inhibition by n-propanol, produced intracellularly from n-propyl gallate. n-Propanol oxidation is probably the main responsible for the increased mitochondrial respiration caused by n-propyl gallate, which is simultaneous to the inhibitory action on the tricarboxylic acid cycle.

Uncoupling of the mitochondrial respiratory chain seems not to be significant at concentrations of n-propyl gallate up to 200 μM in the perfused liver, although it may be significant in incubations with isolated mitochondria. However, higher concentrations, 400 μM for example, are likely to produce significant uncoupling.

As a final conclusion it can be said that n-propyl gallate can be considered a kind of metabolic effector, whose actions on the liver metabolism are relatively mild although they can become harmful for the organ and the whole organism at high doses and concentrations.

The action of n-propyl gallate on gluconeogenesis and oxygen uptake in the rat liver

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Abstract

In the present study the metabolic actions of n-propyl gallate on hepatic gluconeogenesis, oxygen uptake and related parameters were investigated. Experiments were done in the isolated perfused rat liver. n-Propyl gallate inhibited gluconeogenesis and stimulated oxygen uptake at concentrations up to 200 μM. The inhibitory effects on lactate gluconeogenesis (ED_{50} 86.4 μM) and alanine gluconeogenesis were considerably more pronounced than those on glycerol and fructose gluconeogenesis. n-Propyl gallate also stimulated oxygen uptake in both the mitochondrial (63%) and microsomal (37%) electron transport chains. The first one is due mainly to the oxidation of n-propanol, as a metabolite of the first step of n-propyl gallate transformation. The second one results from a direct stimulation of the microsomal electron transport chain. n-Propyl gallate inhibited pyruvate carboxylation (ED_{50} 142.2 μM) in consequence of an inhibition of pyruvate transport into the mitochondria an effect not found for gallic acid. This is probably the main cause for glucose output inhibition. Secondary causes are (1) deviation of intermediates for the production of NADPH to be used in microsomal electron transport; (2) deviation of glucose 6-phosphate for glucuronidation reactions; (3) gluconeogenesis inhibition by n-propanol, produced intracellularly from n-propyl gallate. Inhibition of mitochondrial energy metabolism is not significant in the range up to 200 μM, as indicated by the very small effect on the cellular ATP levels (5% decreased). n-Propyl gallate can be considered a kind of metabolic effector, whose actions on the liver metabolism are relatively mild although they can become harmful for the organ and the whole organism at high doses and concentrations.

Introduction

n-Propyl gallate (n-propyl 3,4,5-trihydroxybenzoate) is an ester formed by the condensation of gallic acid with n-propanol. It has been added to foods (especially oils and fats), cosmetics, hair products, adhesives and lubricants [1] to prevent oxidation. n-Propyl gallate also occurs in many products of plant origin. In green tea, for example, the gallic acid moiety is one of the most common structures among its phenolic compounds, free gallic acid and n-propyl gallate being particularly abundant [2]. Hepatotoxic actions of green tea have been partly attributed to its n-propyl gallate and free gallic acid contents. In mice n-propyl gallate doses of 100 and 200 mg/kg, can increase plasma levels of alanine aminotransferase by 57 and 400%, respectively [2]. In earlier studies with isolated rat liver hepatocytes and mitochondria the actions of high n-propyl gallate concentrations were investigated [1,3]. It was found that n-propyl gallate, at concentrations of 1-2 mM, causes cellular ATP depletion and cell death. These effects can be attenuated by fructose. The authors of these studies [1,3] concluded that the mitochondria are an important target of the toxic action of n-propyl gallate and that this action can be influenced by the cellular energy status. An action on mitochondrial energy metabolism has been reiterated recently in a study in which the actions of several alkyl esters were investigated in mouse hepatocytes, mouse sarcoma 786A and mouse mammary carcinoma TA3 cell lines and its multiresistant variant TA3-MTX-R [4]. The alkyl esters of gallic acid, including n-propyl gallate, inhibit respiration of those cells more effectively than free gallic acid. The order of decreasing potency is n-octyl- ≈ iso-amyl- ≈ n-amyl- ≈ iso-butyl- > n-butyl- > iso-propyl- > n-propyl gallate >> gallic acid. According to the study [4], the alkyl gallates act by blocking the mitochondrial electron flow, especially at the segment NADH-coenzyme Q, impairing ATP synthesis, an event that could lead to cell death. All tumor cells are more sensitive to the alkyl gallates than the mouse hepatocytes. In the various cancer cells n-propyl gallate produces 50% inhibition of respiration at concentrations between 575 and 800 μM; in mouse hepatocytes 50% inhibition can be expected to occur at concentrations above 1.3 mM.

Based on the n-propyl gallate concentrations that are active on mitochondrial respiration of hepatocytes, above 1 mM [2,4], in vivo metabolic effects of the compound can only be expected at very high doses. Nevertheless, it has been recently reported that n-propyl gallate and gallic acid act as free-radical

scavengers at concentrations up to 200 μM [5]. Furthermore, gallic acid inhibits gap-junctional intercellular communication in the same concentration range [5]. It must also be added that hepatic transformation of n-propyl gallate generates n-propanol [3], a compound whose metabolic transformation is similar to that of ethanol, an inhibitor of hepatic gluconeogenesis in consequence of its transformation reactions [6]. All these facts prompted us to investigate possible metabolic actions of n-propyl gallate at concentrations well below 1-2 mM. This purpose can be best achieved by measuring metabolic pathways, such as gluconeogenesis, that are highly sensitive to changes in cell integrity or to relatively small changes in the intracellular concentration of key metabolic intermediates. For measuring gluconeogenesis and associated pathways the isolated perfused rat liver was used. Additionally, sub-cellular fractions, such as microsomes, were used for complementary measurements of several enzymatic activities. The latter should allow to suggest possible mechanisms for the observed effects.

Material and methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. n-Propyl gallate and gallic acid acid and all enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St. Louis, MO,USA). Sodium [^{14}C]bicarbonate (specific activity of 58 Ci/mmol) and labeled octanoate ([1-^{14}C]octanoate), were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were from the best available grade (98–99.8% purity).

Animals

Male albino rats (Wistar), weighing 180–220 g, were fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil). In most experimental protocols, the rats were starved for 24 h before the surgical removal of the liver. All experiments were done in accordance with the internationally accepted recommendations in the care and use of animals.

Liver Perfusion and analytics

For the surgical procedure, the rats were anesthetized by intraperitoneal injection of thiopental (50 mg/kg). Hemoglobin-free, nonrecirculating perfusion was performed [7]. After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 ^{o}C. The flow, provided by a peristaltic pump, was between 30 and 33 mL/min. When perfused with substrate-free perfusion medium livers from 24-hours fasted rats respire mainly at the expense of endogenous fatty acids [7]. In the present work, gluconeogenesis from four different substrates was measured: lactate, alanine, fructose and glycerol.

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were assayed by means of standard enzymatic procedures: glucose, lactate, pyruvate and n-propanol [8]. 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 [7]. Metabolic rates were calculated from input-output differences and the total flow rates and were referred to the wet weight of the liver.

In those experiments in which tracer [1-^{14}C]octanoate was infused for $^{14}CO_2$ production the outflowing perfusate was collected in Erlenmeyer flasks in 2 minute fractions. The Erlenmeyer flasks were rapidly and tightly closed with rubber stoppers to which scintillation vials containing phenylethylamine were fastened by means of stainless steel wires. Trapping of the $^{14}CO_2$ in the phenylethylamine was achieved by acidification of the perfusate with a HCl solution which was injected into the flasks through the rubber stoppers [9]. Radioactivity was measured by liquid scintillation spectroscopy. The scintillation solution was: toluene/ethanol (2/1) containing 5 g/liter 2,5-diphenyloxazole and 0.15 g/liter 2,2-p-phenylene-bis(5-phenyl-oxazole).

The hepatic contents of adenine nucleotides were measured after freeze-clamping the perfused liver with liquid nitrogen. The freeze-clamped livers were extracted with perchloric acid. The extract was neutralized with K_2CO_3 and AMP, ADP, and ATP were assayed by means of standard enzymatic procedures [8].

Cell fractionation procedures

Microsomes were isolated by differential centrifugation [10-12]. Rats were decapitated and their livers excised, cut into small pieces with scissors and washed with a cold (4 ^{o}C) aqueous solution (isolation medium) containing 150 mM KCl, 0.1 mM phenylmethanesulfonylfluoride (PMSF) and 10mM Tris-HCl (pH 7.4). After suspension in 10 volumes of the isolation medium, the tissue was homogenized with a Dounce homogenizer. The homogenate was filtered through gauze and centrifuged at 2550g for 10 min in a refrigerated centrifuge. The supernatant was again centrifuged in two steps of 7100 and 12400g for 10 min. Finally, the supernatant of the last centrifugation was collected and centrifuged at 105000g for 1 h. The pellet containing the microsomal fraction was suspended in cold isolation medium at a final protein concentration of 20 mg protein mL^{-1}.

For mitochondria isolation rats were decapitated, their livers removed immediately and cut into small pieces. The fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 2.0 mM Tris–HCl (pH 7.4), 0.2 mM EGTA, and 50 mg% fatty acid-free bovine-serum albumin. Homogenization was carried out in the same medium by means of a van Potter–Elvehjem homogenizer. After homogenization, the mitochondria were isolated by

differential centrifugation [13,14] and suspended in the same medium, which was kept at 0-4 °C.

Protein content of the microsomal and mitochondrial suspensions was measured using the Folin–phenol reagent and bovine-serum albumin as a standard [15].

Enzyme Assays

Glucose 6-phosphatase was assayed using the microsomal suspension prepared as described above. The incubation medium contained 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.2), 15 mM glucose 6-phosphate, and 0.1–0.2 mg microsomal protein [16,17]. After 20 min incubation at 37 °C, the reaction was stopped by the addition of one volume of 5% trichloroacetic acid and phosphate release was measured [18].

The D-fructose 1,6-bisphosphatase activity was assayed using the supernatant of the 105000g centrifugation obtained during the microsome isolation procedure [17,19]. The reaction mixture contained 0.4–0.8 mg protein/mL, 100 mM Tris–HCl (pH 8), 12 mM $MgCl_2$, 1 mM D-fructose 1,6-bisphosphate, and 5 mM cysteine. After 20 min incubation at 38 °C, the reaction was interrupted by the addition of one volume of 5% trichloroacetic acid and phosphate release was measured [18].

The pyruvate carboxylase activity of intact mitochondria was assayed by measuring the incorporation of ^{14}C from [^{14}C]$NaHCO_3$ into components of the tricarboxylic acid cycle. The incubation medium contained 5 mM pyruvate, 12.5 mM $MgCl_2$, 2.5 mM potassium phosphate, 120 mM KCl, 10 mM HEPES (pH7.5), and 3 mg protein/mL [20]. The reaction was initiated by introducing 12 mM [^{14}C]$NaHCO_3$ (0.25 μCi). After 10 min of incubation at 37 °C, the reaction was arrested by the addition of a 0.5 volume of 2 N perchloric acid. After expulsion of the remaining [^{14}C]$NaHCO_3$ (5 min), aliquots were taken for counting the acid stable incorporated radioactivity. The pyruvate carboxylase of disrupted mitochondria was measured using a medium able to generate steady-state concentrations of acetyl-CoA [21]. Rat liver mitochondria, isolated as described above, were disrupted by successive freeze and thawing procedures using liquid nitrogen. The incubation medium contained 3 mg protein/mL of disrupted mitochondria, 5 mM sodium pyruvate, 12.5 mM $MgCl_2$, 2.5 mM potassium phosphate, 0.3 M sucrose, 1 mM ethylenediamine tetraacetate, 5 mM tris(hydroxymethyl)aminomethane (TRIS; pH 7.5), 0.5 mM lithium coenzyme A,

5 mM adenosine triphosphate, 1.1 mM acetyl phosphate, 1 unit/mL phospho-transacetylase and 1 unit/mL citrate synthase. The reaction was initiated by introducing 12 mM [^{14}C]$NaHCO_3$ (0.25 μCi). After 10 min of incubation at 37 °C, the reaction was arrested by the addition of 0.5 volume of 2 N perchloric acid. After expulsion of the remaining [^{14}C]$NaHCO_3$ (5 min), aliquots were taken for counting the acid stable incorporated radioactivity. The incorporated radioactivity in both incubations, intact and disrupted mitochondria, was expressed as nmol min^{-1} mg $protein^{-1}$. The scintillation solution for counting ^{14}C was composed of toluene/Triton X-100® (1.5/0.5), 10 g/L 1,5-diphenyloxazole plus 0.4 g/L 2,2-*p*-phenyl-bis-5-phenyleneoxazole.

Mitochondrial and microsomal oxygen consumption

Oxygen uptake by isolated mitochondria or microsomes was measured polarographically using a Teflon-shielded platinum electrode [12,22]. Intact mitochondria (2 mg protein/mL) were incubated in the closed oxygraph chamber at 36 °C in a medium (2.0 mL) containing 0.25 M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM EDTA, 25 mg% fatty acid-free bovine-serum albumin, 10 mM Tris–HCl (pH 7.4), and substrates in addition to various n-propyl gallate acid concentrations in the range up to 200 μM. The substrates were succinate (7.5 mM) and α-ketoglutarate (7.5 mM); ADP was added for a final concentration of 0.125 mM. Rates of oxygen uptake were computed from the slopes of the recorder tracings and expressed as nmol min^{-1} mg $protein^{-1}$.

Isolated microsomes (4 mg protein/mL) were incubated in the closed oxygraph chamber at 36 °C in a medium (2.0 mL) containing 0.25 M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM EDTA, 25 mg% fatty acid-free bovine-serum albumin, 10 mM Tris–HCl (pH 7.4). Oxygen uptake was started by the addition of 1 mM NADPH, which was followed by the addition of up to 600 μM n-propyl-gallate or gallic acid. Rates of oxygen uptake were computed from the slopes of the recorder tracings and expressed as nmol min^{-1} mg $protein^{-1}$.

Treatment of data

The statistical significance of the differences between parameters obtained in the experiments was evaluated by means of Student's *t* test, and Student's paired t test according to the context. The results are discussed in the text using *p* values; $p < 0.05$ was the criterion of significance. Concentrations for half-

maximal effects were determined by numerical interpolation using the Scientist software from MicroMath Scientific Software (Salt Lake City, UT).

Results

The first experiments were done in order to test the activity of n-propyl gallate on gluconeogenesis. Figure 1 shows the time course of perfusion experiments in which 100 μM n-propyl gallate was infused in the presence of lactate as gluconeogenic precursor. It illustrates the general protocol that was also used with other substrates. Glucose output before lactate infusion was minimal because livers from 24-hours fasted rats possess minimal levels of glycogen and also because the concentrations of endogenous substrates are very small. The introduction of lactate immediately raised glucose production as well as oxygen consumption and pyruvate production. Infusion of 100 μM n-propyl gallate (at 40 minutes perfusion time) clearly decreased glucose output (55%) with a simultaneous increase in oxygen uptake. Pyruvate production suffered a slow increase which still continued after cessation of n-propyl gallate infusion. Glucose production and oxygen uptake, however, tended to return to the levels previous to n-propyl gallate introduction when the infusion of this substance was interrupted (at 60 minutes perfusion time). The introduction of n-propyl gallate in the perfusion system without liver did not produce any perceptible change in the outflowing oxygen concentration. This control experiment excludes the possibility of a significant non-enzymatic oxidation of n-propyl gallate during a single passage through the liver [3]. The same kind of experiment was done with gallic acid, and the results are shown in Figure 2. Gallic acid (100 μM) also decreased glucose output, but to a much smaller extent than n-propyl gallate (20%). Oxygen uptake was not significantly increased but there was a small increase in pyruvate production.

Experiments like those illustrated by Figure 1 were repeated with several n-propyl gallate concentrations. The results are summarized in Figure 3 which shows the changes caused by n-propyl gallate as a function of its concentration in the portal vein. For calculating these changes the rates in the presence of substrate + n-propyl gallate were subtracted from the rates in the presence of substrate alone. Gluconeogenesis diminution shows a clear concentration dependence; numerical interpolation revealed 50% inhibition at a concentration of 86.4 μM. Oxygen uptake stimulation presents saturation; numerical interpolation predicts half-maximal stimulation at a concentration of 42.8 μM. Pyruvate production shows saturation in the range up to 100 μM and increases further in the passage from 100 to 200 μM.

The experiments that were done with lactate as gluconeogenic substrate were repeated with three additional substrates namely alanine, fructose and glycerol. In these experiments 200 μM n-propyl gallate was infused. The results of these experiments are summarized in Table 1. The results obtained with lactate are also listed for comparative purposes. With 200 μM n-propyl gallate the inhibition of gluconeogenesis from lactate was 80%, but it was 99% when alanine was the precursor. With the precursors fructose and glycerol the inhibition was less pronounced, 30 and 27%, respectively. The most pronounced relative stimulation of oxygen uptake by n-propyl gallate was that one found with glycerol as the substrate: 9.5 fold in relation to the stimulation caused by the substrate alone. This is much more than that found with lactate, for example, for which a 1.3 fold increase was found. Pyruvate productions from all substrates except fructose were stimulated by n-propyl-gallate. And, finally, lactate productions from alanine and fructose were also stimulated.

The activity of the tricarboxylic acid cycle was quantified as $^{14}CO_2$ production after labelling the cellular acetyl-CoA pools with the infusion of tracer amounts of [1-^{14}C]octanoate. The infusion of tracer amounts of labelled octanoate do not produce any net change in the metabolic fluxes (oxygen uptake, for example) [23,24]. Figure 4 shows the time course of the corresponding experiments. As expected, no changes were found in oxygen uptake in consequence of [1-^{14}C]octanoate infusion; the $^{14}CO_2$ production, however, increased rapidly to steady-state levels due to the rapid labelling of the acetyl-CoA pools. The introduction of n-propyl gallate produced the already known increment in respiration and a clear and reversible inhibition of $^{14}CO_2$ production.

The increment in respiration caused by n-propyl gallate could be due to stimulation of the mitochondrial respiratory chain, to stimulation of the microsomal electron transport system or to both. Attempting to discriminate between these two possibilites, experiments were done in which the mitochondrial respiratory chain of perfused livers was blocked by the infusion of 2 mM sodium cyanide. Substrate-free perfused livers were utilized. As expected, cyanide produced a substantial reduction of respiration (from 1.56±0.10 to 0.66±0.11 μmol min^{-1} g^{-1}). Under these conditions oxygen uptake stimulation by n-propyl gallate was substantially reduced (63%), as can be deduced from data in Table 2. Comparatively, as also shown in Table 2, the same cyanide concentration virtually abolished the stimulating action of n-propanol (200 μM) and 2,4-dinitrophenol, the latter being an uncoupler of the mitochondrial

respiratory chain (100 μM). Total abolition of the capacity of n-propyl gallate as an oxygen uptake stimulator was obtained, however, by the simultaneous infusion of 2 mM cyanide and 100 μM proadifen, the latter being an inhibitor of the microsomal electron transport chain.

n-Propanol is probably the first product of n-propyl gallate metabolism [3]. It is also a compound for which metabolic effects can be expected, as already evidenced by the observed increment in oxygen uptake shown in Table 2. For this reason attempts were made to quantify n-propanol in the outflowing perfusate. Only traces of n-propanol were found in the outflowing perfusate during 200 μM n-propyl gallate infusion. At the concentrations of 500 and 1000 μM, however, n-propanol appeared in substantial amounts, as shown in Figure 5. It is important to note that the n-propanol overflow increased by a factor of approximately 4.3 when the portal n-propyl gallate concentration was doubled (500 to 1000 μM). The action of n-propanol on gluconeogenesis was also examined because it is known that ethanol inhibits this metabolic pathway in consequence of its metabolic transformation [6]. At the concentration of 200 μM n-propanol clearly inhibited lactate gluconeogenesis from 0.808±0.051 to 0.639±0.031 μmol min^{-1} g^{-1} (21%) without changing, under these conditions, oxygen uptake.

n-Propyl gallate up to 200 μM did not increase respiration driven by succinate and α-ketoglutarate in intact mitochondria either in the absence or presence of exogenously added ADP (not shown). This confirms previous reports [3]. Also confirming previous reports [3], the respiratory control index was clearly impaired by n-propyl gallate with both substrates. With succinate, 200 μM n-propyl reduced the respiratory control index from 5.3 to 3.91; with α-keto-glutarate as a substrate the reduction was more pronounced, from 4.0 to 2.0.

The action of n-propyl gallate on the microsomal electron transport was investigated by incubating isolated microsomes with NADPH. Addition of NADPH initiated electron transfer to oxygen without further additions probably due to the hydroxylation of endogenous substrates. This basal rate was equal to 2.77±0.04 nmol min^{-1} mg $protein^{-1}$. The subsequent addition of n-propyl gallate, however, produced a clear concentration dependent stimulation, which is represented in Figure 6. The graph shows saturation for the n-propyl gallate stimulatory effect with 50% of maximal stimulation at the concentration of 37.5 μM. When the same experiments were done with gallic acid in the same concentration range, no stimulation of the NADPH-driven microsomal oxygen uptake was found.

Diminution of the respiratory control index in isolated mitochondria [3] is indicative of impaired mitochondrial ATP generation. If this really occurs in the perfused liver can be tested by measuring the cellular levels of adenine nucleotides. In the search for a possible correlation between inhibition of gluconeogenesis and impairment of ATP production the measurements were conducted under gluconeogenic conditions. The results are listed in Table 3 which reveals that the tissue levels of AMP and ADP were not affected by n-propyl gallate. There was, however, a small (5%), though statistically significant, reduction in the tissue levels of ATP.

Three key enzymatic activities of the gluconeogenic pathway were analyzed in the present work: pyruvate carboxylase, glucose 6-phosphatase and fructose 1,6-bisphosphatase. The results of several measurements at various n-propyl gallate concentrations are shown in Figures 7 and 8. Pyruvate carboxylation in isolated intact mitochondria was inhibited by n-propyl gallate with a well defined concentration dependence (Figure 7). Half-maximal inhibition was found for a concentration of 142.2 μM. Inhibition of the pyruvate carboxylase activity of freeze-thawing disrupted mitochondria was minimal. Gallic acid, on the other hand, did not inhibit the pyruvate carboxylase activity of intact or disrupted mitochondria. No action of n-propyl gallate was found on glucose 6-phosphatase and fructose 1,6-bisphosphatase (Figure 8), at least not in the concentration range up to 400 μM.

Discussion

The most important observation of this work is that n-propyl gallate is able to affect the metabolism of the rat liver without cellular ATP depletion at concentrations considerably smaller than those reported to be inhibitory for the mitochondrial energy metabolism in isolated hepatocytes. The interactions of n-propyl gallate with the liver are relatively complex and involve direct effects as well as effects derived from its transformation reactions, especially the intracellular release of n-propanol which is further metabolized. Figure 9 shows a scheme that allows to visualize the main points to be discussed below.

The most prominent effect is inhibition of glucose output, but the compound is able to produce several alterations in the hepatocyte in terms of metabolic fluxes. The most important cause for the diminution of glucose output seems to be inhibition of gluconeogenesis in consequence of an inhibition of pyruvate carboxylation. This, in turn, results from an inhibition of pyruvate transport across the mitochondrial membrane (see Figure 9), as indicated by the observation that inhibition of carboxylation is solely significant in intact mitochondria. That inhibition of pyruvate transport into the mitochondria diminishes glucose synthesis from substrates that depend on such a transport has been amply demonstrated in experiments with the classical inhibitor of pyruvate transport α-cyano-4-hydroxycinnamic acid [25] and also in experiments with *p*-coumaric acid [17]. Both lactate (lactate dehydrogenase) and alanine (alanine aminotransferase) must be first transformed into pyruvate before they enter the gluconeogenic pathway (Figure 9). Although a mitochondrial form of alanine aminotransferase also exists, in the rat liver the ratio of the cytosolic to the mitochondrial activity is equal to 5.12 [26]. Inhibition of pyruvate transport into the mitochondria is probably also responsible for the observed increases in pyruvate or lactate output observed in the present work. The release of these metabolites must be lumped together because of the lactate dehydrogenase equilibrium (see Figure 9), their relative proportions closely reflecting the cytosolic NADH/NAD^+ ratio under the various experimental conditions [7,27].

Inhibition of pyruvate carboxylation, however, is not the only mechanism underlying inhibition of glucose output by n-propyl gallate. This is indicated by the fact that the compound also inhibits, though less strongly, glucose output driven by precursors (fructose and glycerol) whose entry into the gluconeogenic pathway is situated after the pyruvate carboxylase reaction. In this respect there

are several mechanistic possibilities as illustrated in Figure 9: (a) deviation of a fraction of glucose 6-phosphate into the pentose-monophosphate shunt for the production of reducing equivalents in the form of NADPH to be used in the n-propyl gallate-stimulated microsomal electron transport [28]; (b) deviation of a fraction of glucose 6-phosphate for glucuronidation reactions [29]; (c) inhibition of gluconeogenesis by deviating cytosolic malate (in equilibrium with oxalo-acetate) for the production of NADPH for microsomal electron transport [28]; (d) gluconeogenesis inhibition by n-propanol, produced intracellularly from n-propyl gallate [6]; n-propanol oxidation is probably the main responsible for the in-creased mitochondrial respiration caused by n-propyl gallate, which is simul-taneous to the inhibitory action on the tricarboxylic acid cycle (see Figure 9). In this respect it should be considered that the relation between the intracellular propanol production and the portal n-propyl gallate concentration, as all enzymic processes, is a saturable phenomenon. The propanol output, however, behaved differently in that it accelerated continuously starting from zero at portal n-propyl gallate concentrations below 200 μM. This set of observations indicates that with portal n-propyl gallate concentrations up to 200 μM all propanol produced was transformed intracellularly by the usual pathways that lead to the production of propionic acid and succinate [30,31]. Direct inhibition of enzymatic steps of the gluconeogenic pathway in addition to the three that were measured in the present work should not be completely excluded. However, it must be stressed that unless such a hypothetical inhibitory effect is very strong, its contribution will be quite small. For lactate gluconeogenesis, for example, pyruvate carboxylase is by far the most important regulatory enzyme, as can be deduced from its flux control coefficient (between 56 and 75%) that greatly surpasses those ones of the other enzymes including phosphoenolpyruvate carboxykinase (between 0.2 and 5%) [32]. Finally, uncoupling of the mitochondrial respiratory chain seems not to be significant at concentrations of n-propyl gallate up to 200 μM in the perfused liver, although it may be significant in incubations with isolated mitochondria, because only a very small diminution (5%) of cellular ATP was found. However, higher concentrations are likely to produce significant uncoupling.

The deviation of a fraction of glucose 6-phosphate or L-malate for the production of reducing equivalents in the form of NADPH is a likely phenomenon because stimulation of microsomal electron transport by n-propyl gallate was found to occur in both perfused liver and isolated microsomes (Figure 9).

Although it seems of secondary importance in the case of n-propyl gallate, for other gluconeogenesis inhibitors such as aminopyrine it is of primary importance [28,29]. Deviation of glucose 6-phosphate into the pentose-monophosphate shunt for the production of NADPH will produce a net reduction in glucose output even when fructose and glycerol are the substrates. Transformation of L-malate into pyruvate with transfer of the reducing equivalents to $NADP^+$ will further reduce gluconeogenesis from alanine and lactate. Conjugation of free gallic acid or even n-propyl gallate to glucuronic acid [3] can also deviate glucose 6-phosphate with a diminution of glucose output. Normally the main source of glucuronic acid is glycogenolysis [29], but in the virtual absence of glycogen in livers from fasted rats [33], gluconeogenesis is the sole possible source [28]. It should be stressed that gallic acid does not enhance microsomal respiration. Consequently, with the data available so far, deviation of glucose 6-phosphate for glucuronidation reactions is the only explanation for the small diminution of glucose output caused by this compound.

That n-propyl gallate stimulates microsomal electron transport toward oxygen was substantiated by experiments with the perfused liver and with isolated microsomes. Blocking of the mitochondrial respiratory chain by 2 mM cyanide was apparently complete as indicated by the fact that no stimulation of oxygen uptake occurred upon 2,4-dinitrophenol infusion. The remaining stimulatory capacity of n-propyl gallate, on the other hand, was totally sensitive to proadifen, a well known inhibitor of the microsomal electron transport mediated by cytochrome P_{450} [34]. Also, n-propanol infusion did no longer stimulate oxygen uptake in the presence of cyanide suggesting that stimulation of microsomal respiration by n-propyl gallate in the intact cell is produced by the compound itself. This is further corroborated by the absence of stimulation of microsomal electron transport by gallic acid. The latter observation, in turn, strongly suggests that n-propyl gallate is not participating as a substrate in the reactions that accompany the microsomal NADPH oxidation. n-Propyl gallate is most probably acting as a stimulating agent in the oxidation (hydroxylation) of other endogenous substrates which are certainly present in reasonable amounts in the intact cell and also in the microsomal membrane preparations.

As a final conclusion it can be said that n-propyl gallate can be considered a kind of metabolic effector, whose actions on the liver metabolism are relatively mild although they can become harmful for the organ and the whole organism at very high doses and concentrations. In this respect it can also be remembered

that both gallic acid and n-propyl gallate act as free-radical scavengers in the same concentration range in which the latter exerts the metabolic effects reported in the present work [5]. Furthermore, gallic acid, but not n-propyl gallate, inhibits gap-junctional intercellular communication via phosphorylation of connexin 43 and extracellular-signal-regulated kinase1/2 in rat liver epithelial cells [5]. It remains to be investigated how these effects of gallic acid fit into the overall picture of the n-propyl gallate metabolic effects, especially in the presence of hormones and other circulating effectors. This is of especial interest if one remembers that inhibition of pyruvate transport into the mitochondria can affect cholesterol and fatty acid synthesis [35], also processes that are under complex hormonal control.

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Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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Table 1

Influence of n-propyl gallate on gluconeogenesis from various substrates and related metabolic fluxes. Liver perfusion experiments were done with several substrates according to the experimental protocol illustrated by Figures 1 and 2. The basal rates (before starting the infusions) were subtracted from the steady-state metabolic fluxes in the presence of substrate alone and from those in the presence of substrate plus n-propyl gallate. The data represent means ± mean standard errors. The supercripts refer to statistical analysis (Student's t test), whose results are given at the bottom.

Inflowing perfusate conditions	Glucose production	Oxygen uptake increment	Pyruvate production	Lactate production
	μmol min^{-1} g^{-1}			
Lactate (2 mM)	0.730±0.013[a]	0.550±0.064[b]	0.386±0.049[c]	–
Lactate (2 mM) + n-propyl gallate (200 μM)	0.146±0.009[a]	0.720±0.072[b]	0.493±0.056[c]	–
Alanine (2.5 mM)	0.325±0.014[d]	0.533±0.110[e]	0.088±0.006[f]	0.261±0.037[g]
Alanine (2.5 mM) + n-propyl gallate (200 μM)	0.001±0.011[d]	0.731±0.095[e]	0.273±0.004[f]	0.442±0.063[g]
Glycerol (2.5 mM)	0.397±0.036[h]	0.045±0.035[i]	0.001±0.001[j]	0.151±0.014[k]
Glycerol (2.5 mM) + n-propyl gallate (200 μM)	0.290±0.029[h]	0.428±0.018[i]	0.135±0.020[j]	0.181±0.026[k]
Fructose (2.5 mM)	1.476±0.189[ℓ]	0.501±0.048[m]	0.353±0.025[n]	0.959±0.115[o]
Fructose (2.5 mM) + n-propyl gallate (200 μM)	1.034±0.151[ℓ]	0.713±0.021[m]	0.339±0.004[n]	1.258±0.152[o]

[a]$p < 0.001$ (n=3); [b]$p < 0.001$ (n=7); [c]$p < 0.001$ (n=7); [d]$p < 0.001$ (n=3); [e]$p = 0.006$ (n=3); [f]$p = 0.002$ (n=3); [g]$p = 0.031$; [h]$p < 0.001$ (n=4); [i]$p < 0.001$ (n=4); [j]$p = 0.004$ (n=4); [k]$p = 0.069$ (n=4); [ℓ]$p = 0.005$ (n=4); [m]$p = 0.007$ (n=4); [n]$p = 0.57$ (n=4); [o]$p = 0.009$ (n=4).

Table 2

Changes in hepatic oxygen uptake caused by n-propyl gallate and other compounds with or without blockage of the respiratory chain with 2 mM cyanide. Livers were perfused in an open system as described in Materials and Methods using substrate-free perfusion medium. Cyanide (2 mM) was infused during 16 minutes with the subsequent infusion of the substances listed in the first column during 20 minutes. Controls were done without previous cyanide infusion. The oxygen uptake changes due to each substance or combination of substances were calculated as [rate of O_2 consumption after 20 minutes infusion] – [rate of oxygen uptake before the infusion]. Each datum represents the mean ± mean standard errors of 3 liver perfusion experiments.

Inflowing perfusate conditions	**Oxygen uptake changes** $\mu mol\ O_2\ min^{-1}\ g^{-1}$	
	No cyanide (controls)	2 mM cyanide
200 μM n-propyl gallate	+0.330±0.02	+0.121±0.011
100 μM proadifen + 200 μM n-propyl gallate	—	+0.010±0.020
100 μM 2,4 dinitrophenol	+0.831±0.062	–0.010±0.010
200 μM n-propanol	+0.337±0.049	+0.002±0.038

Table 3

Influence of n-propyl gallate on the adenine nucleotide contents of perfused rat livers. Livers were perfused in an open system as described in Materials and Methods. The protocol illustrated by Figure 1 was followed. Livers were freeze clamped with liquid nitrogen at 40 minutes (30 minutes lactate infusion) and at 60 minutes perfusion time (20 min after starting n-propyl gallate infusion). The adenine nucleotides were extracted with cold perchloric acid. Determination was accomplished by standard enzymatic procedures. The super-scripts refer to statistical analysis (Student's t test), whose results are given at the bottom.

Inflowing perfusate conditions	ATP	ADP	AMP
	μmol per gram liver wet weight		
2 mM lactate (n= 3)	1.64±0.01[a]	0.50±0.04[b]	0.19±0.01[c]
2 mM lactate + n-propyl Gallate (200 μM) (n = 4)	1.56±0.02[a]	0.52±0.05[b]	0.20±0.01[c]

[a]p = 0.024; [b]p = 0.78; [c]p = 0.52.

Figure 1

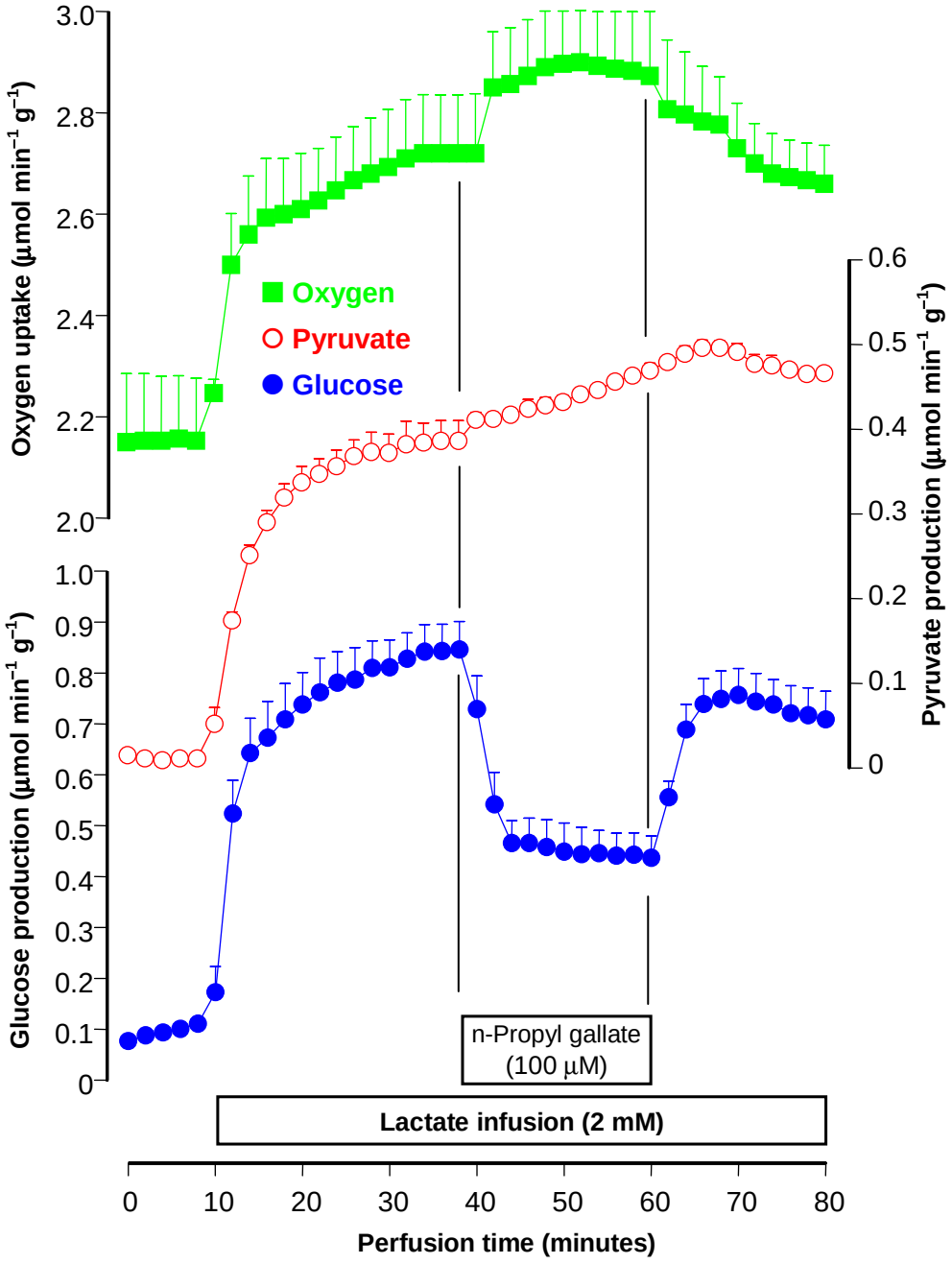

Figure 1**. Time course of the effects of 100 µM n-propyl gallate on metabolic fluxes derived from lactate metabolism in livers from fasted rats.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in the Materials and Methods section. Samples of the effluent perfusate were withdrawn for glucose and pyruvate assay. Oxygen in the venous perfusate was monitored polarographically. The lactate and n-propyl gallate infusion times are indicated by horizontal bars. The data represent the means (±SEM) of three liver perfusion experiments.

Figure 2

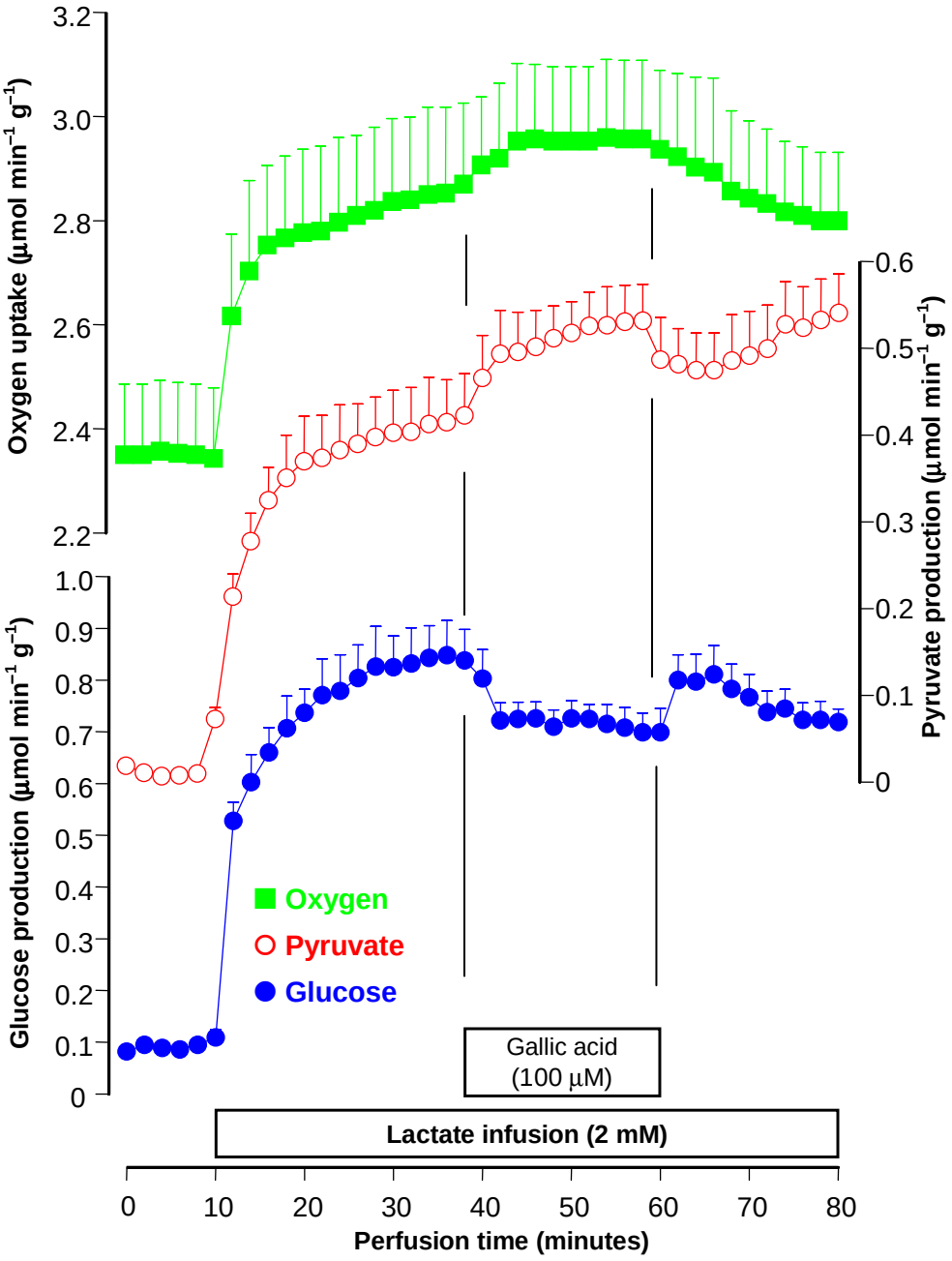

Figure 2**. Time course of the effects of 100 µM gallic acid on metabolic fluxes derived from lactate metabolism in livers from fasted rats.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in the Materials and Methods section. Samples of the effluent perfusate were withdrawn for glucose and pyruvate assay. Oxygen in the venous perfusate was monitored polarographically. The lactate and gallic acid infusion times are indicated by horizontal bars. The data represent the means (±SEM) of three liver perfusion experiments.

Figure 3

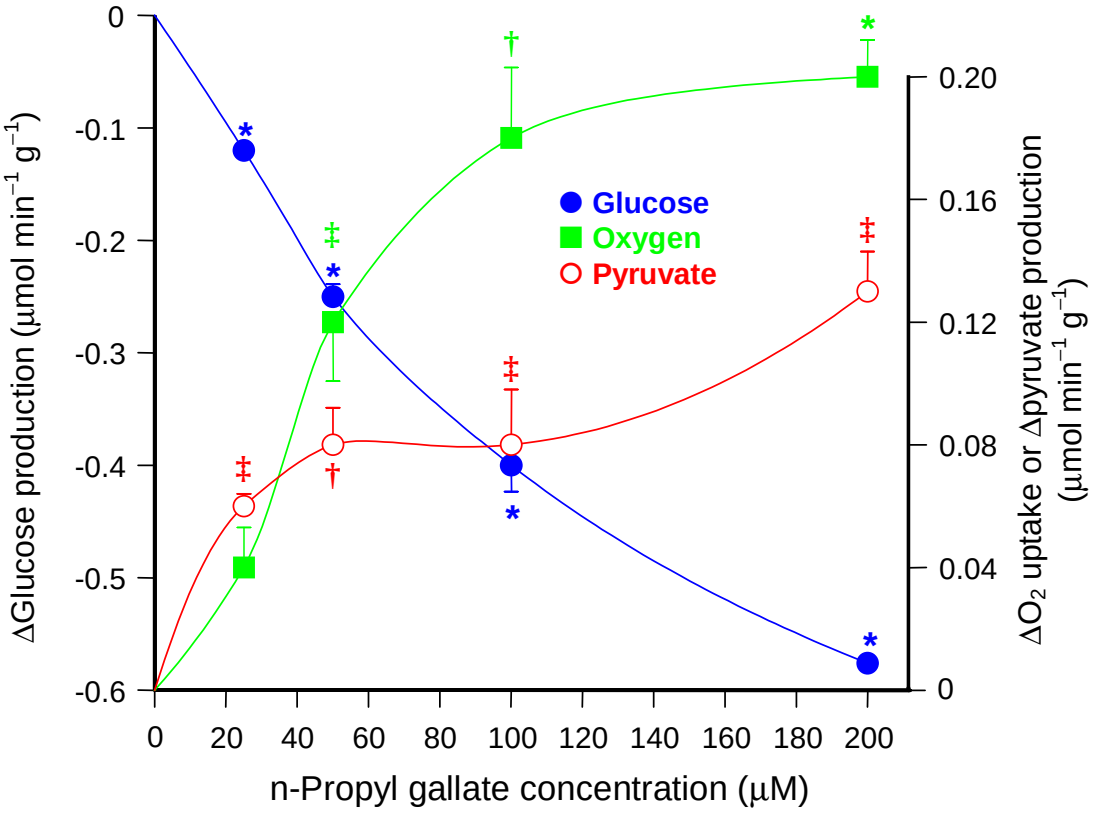

Figure 3**. Concentration dependences of the changes caused by n-propyl gallate on lactate metabolism in the perfused rat liver.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) according to the protocol illustrated by Figure 1 using various n-propyl gallate concentrations. The changes caused by n-propyl gallate were evaluated and represented against the n-propyl gallate concentration. These changes were calculated as the difference between the rates in the presence of lactate alone and the maximal (or minimal) rate after the onset of n-propyl gallate infusion. The data points represent the means ± mean standard errors of 3-7 liver perfusion experiments. Statistical significance was evaluated by applying Student's paired t test. The results are indicated by the symbols *, † and ‡ meaning, respectively, $p \leq 0.01$, $p \leq 0.02$ and $p \leq 0.05$.

Figure 4

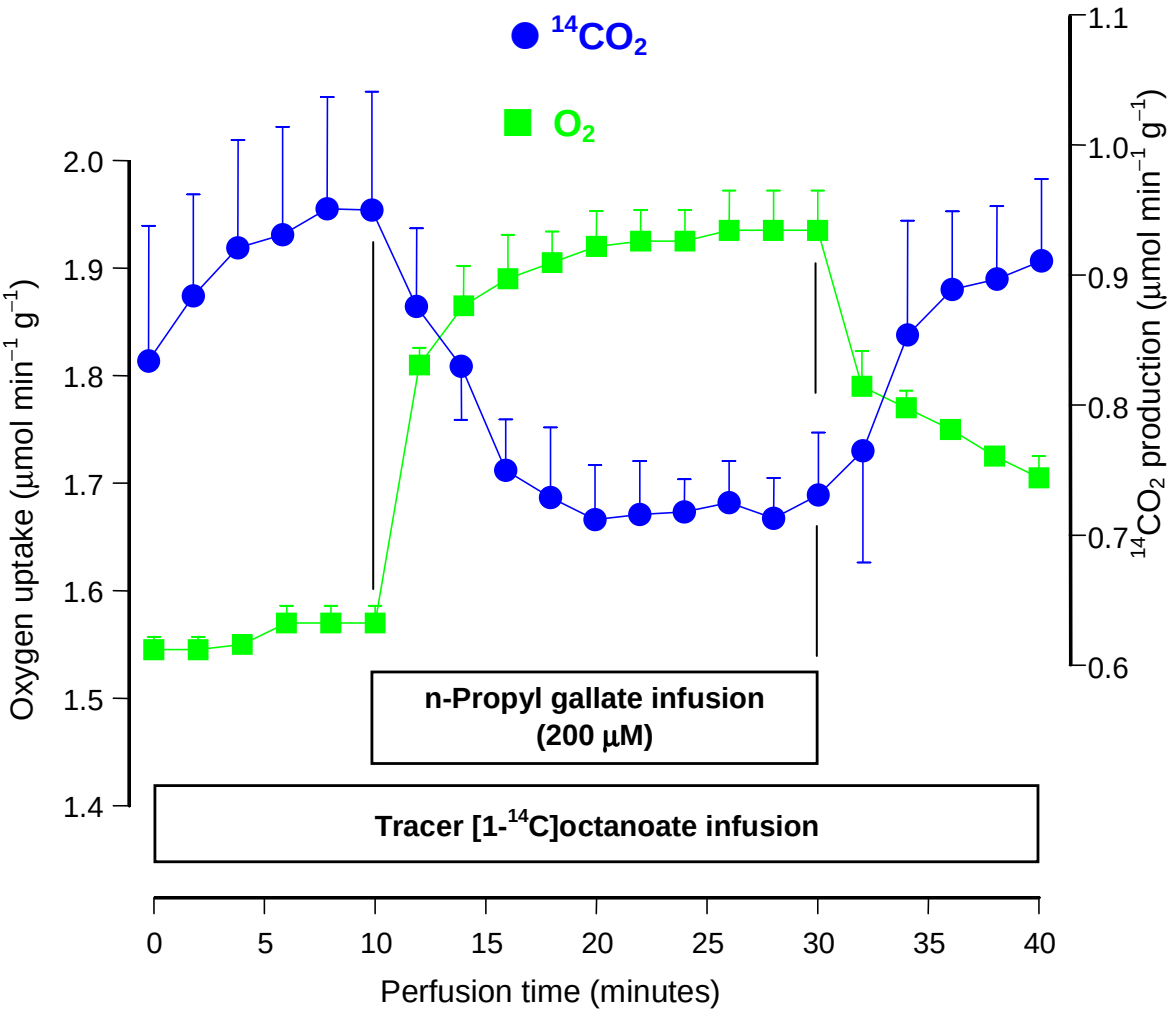

Figure 4**. Time course of the effects of 100 µM n-propyl gallate on oxygen uptake and $^{14}CO_2$ production.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in the Materials and Methods section. Tracer [1-^{14}C]octanoate was infused as indicated, followed by n-propyl gallate infusion. Oxygen uptake was monitored polarographically. The $^{14}CO_2$ in the outflowing perfusate was trapped in phenylethylamine as described in Materials and Methods and the radioactivity was counted by means of liquid scintillation spectrometry. Data are the means ± mean standard errors of three liver perfusion experiments.

Figure 5

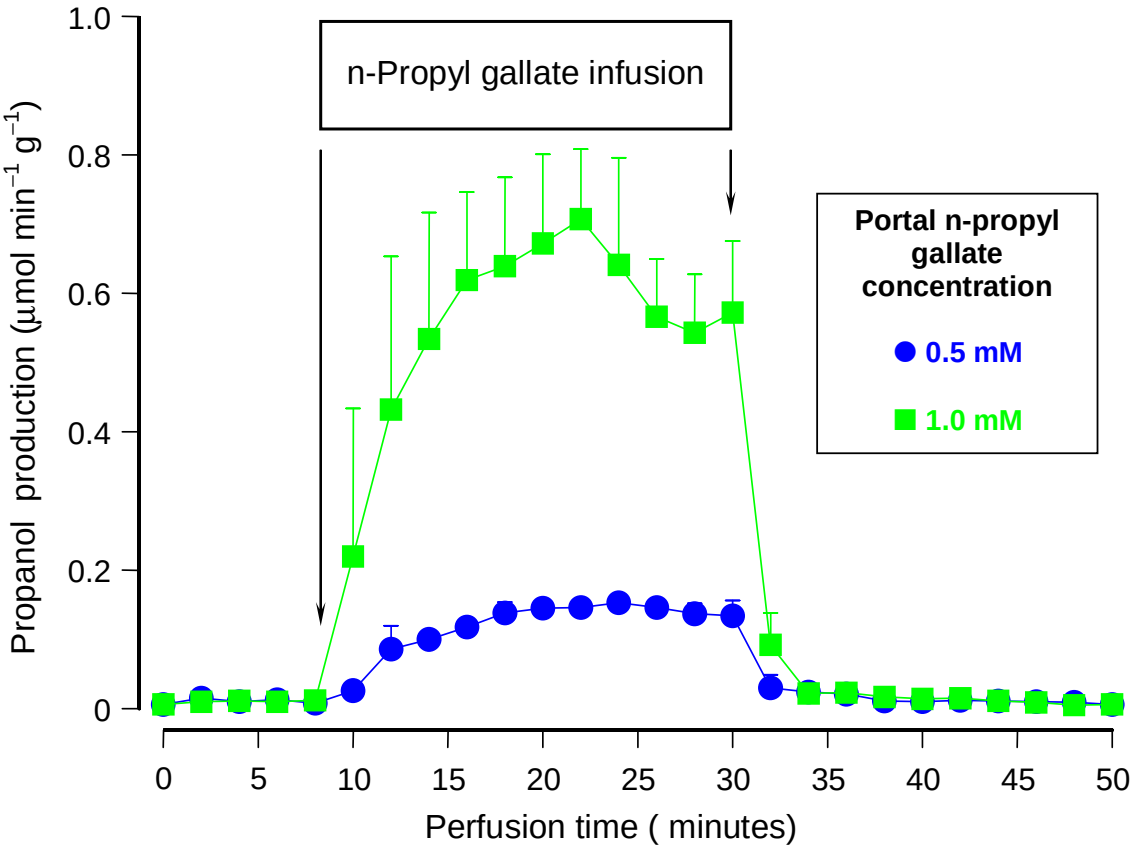

Figure 5**. Time course of n-propanol release upon n-propyl gallate infusion in the perfused rat liver.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in the Materials and Methods section. n-Propyl gallate was infused as indicated and samples of the outflowing perfusate were collected for n-propanol measurement. Data are the means ± mean standard errors of 3 liver perfusion experiments.

Figure 6

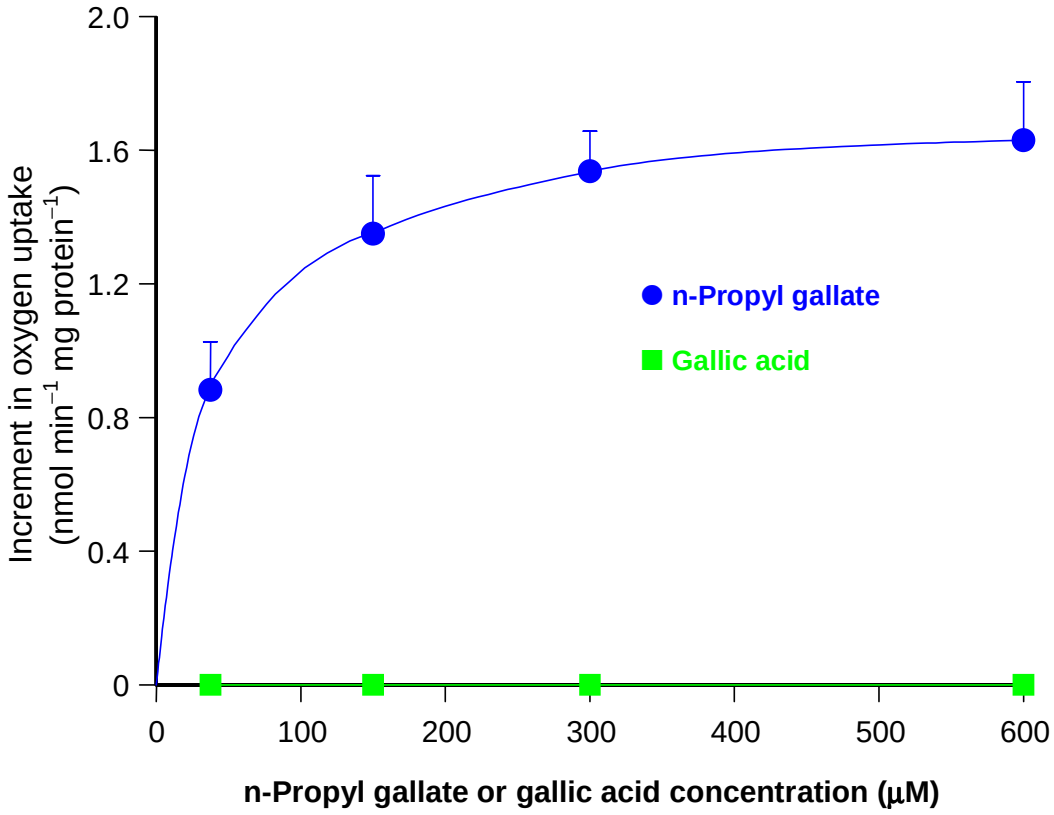

Figure 6**. Increments in oxygen uptake in isolated microsomes caused by n-propyl gallate.** Microsomes were isolated as described in the Materials and Methods section and were incubated in the closed oxygraph chamber at 36 °C in a medium (2.0 mL) containing 0.25 M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM EDTA, 25 mg% fatty acid-free bovine-serum albumin, 10 mM Tris–HCl (pH 7.4) and microsomes (4.0 mg protein/mL). Oxygen uptake was started by the addition of 1 mM NADPH, which was followed by the addition of n-propyl gallate or gallic acid. Rates of oxygen uptake were computed from the slopes of the recorder tracings. The increments were calculated by subtracting the rates in the presence of NADPH alone from the rates in the presence of n-propyl gallate or gallic acid.

Figure 7

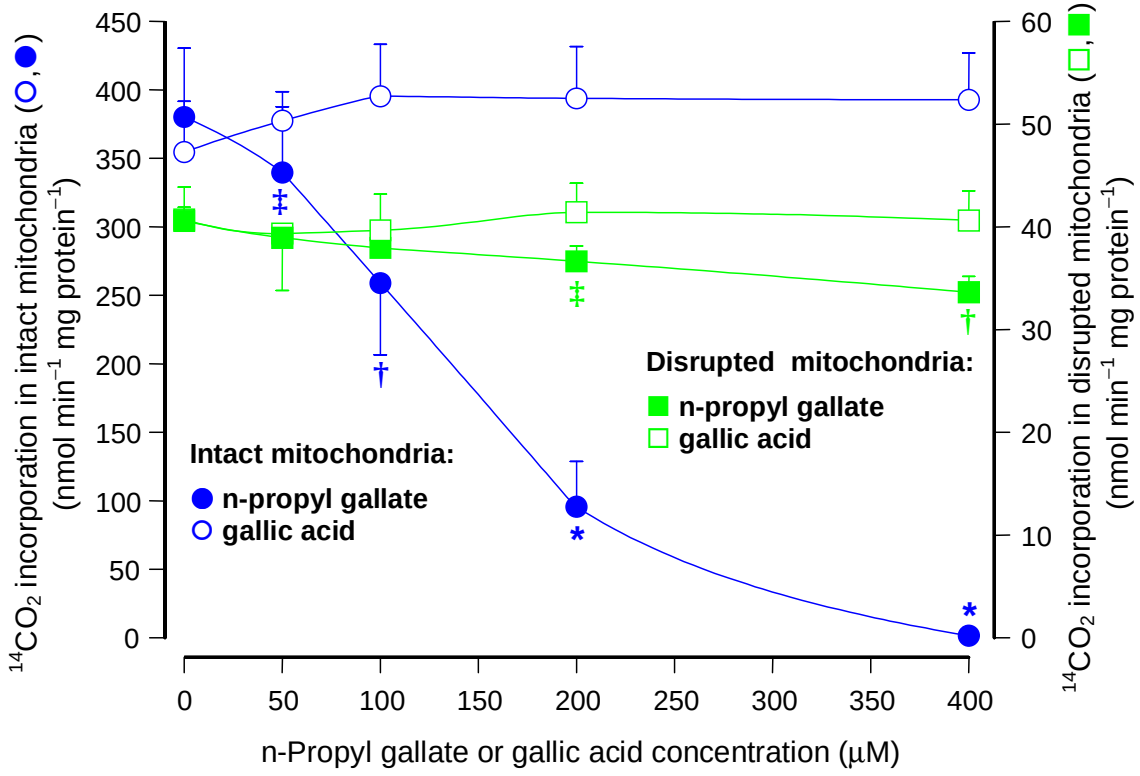

Figure 7**. The pyruvate carboxylase activities of intact and disrupted rat liver mitochondria at various n-propyl gallate and gallic acid concentrations.** The $^{14}CO_2$ incorporation into non-volatile components of the citric acid cycle was measured as described in the Materials and Methods section. Each data point represents the mean ± mean standard error of 4 determinations. Statistical significance was evaluated by applying Student's paired t test (control values versus n-propyl gallate or gallic acid values). The results are indicated by the symbols *, † and ‡ meaning, respectively, $p \leq 0.01$, $p \leq 0.02$ and $p \leq 0.05$.

Figure 8

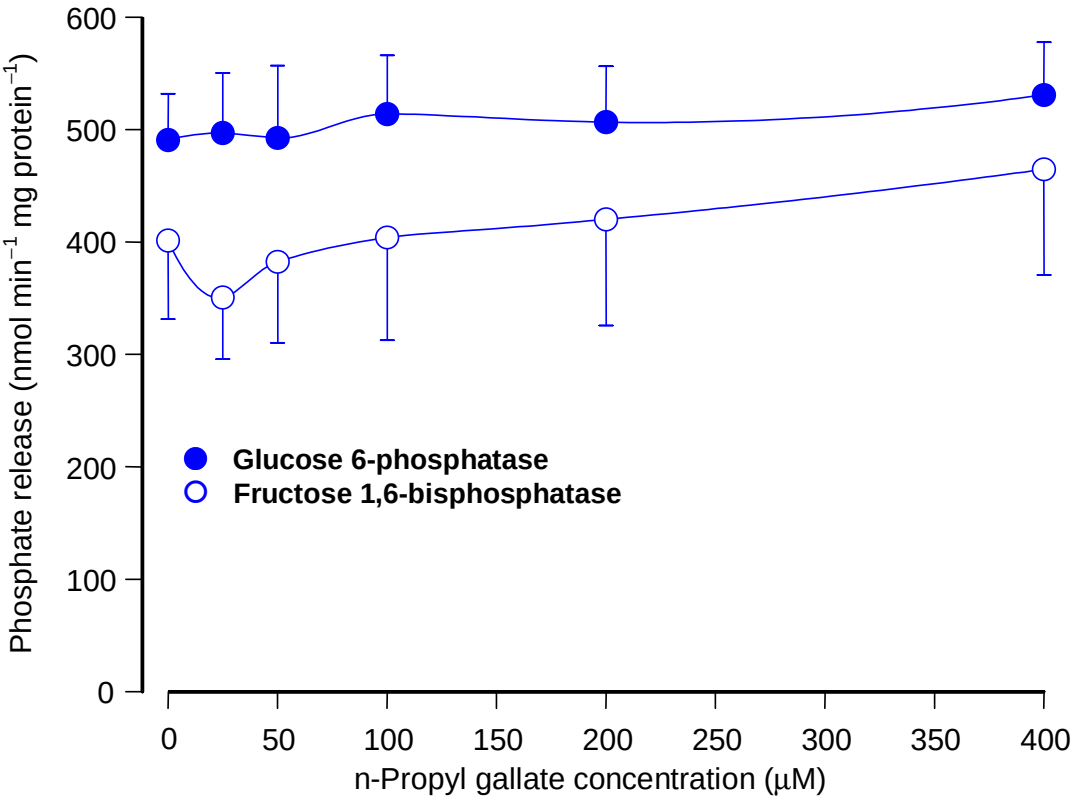

Figure 8**. Glucose 6-phosphatase and fructose 1,6-bisphosphatase activities at several n-propyl gallate concentrations.** Phosphate release from glucose 6-phosphate and fructose 1,6-bisphosphate was measured as described in the Materials and Methods section. Each data point represents the mean ± mean standard error of 4 determinations.

Figure 9

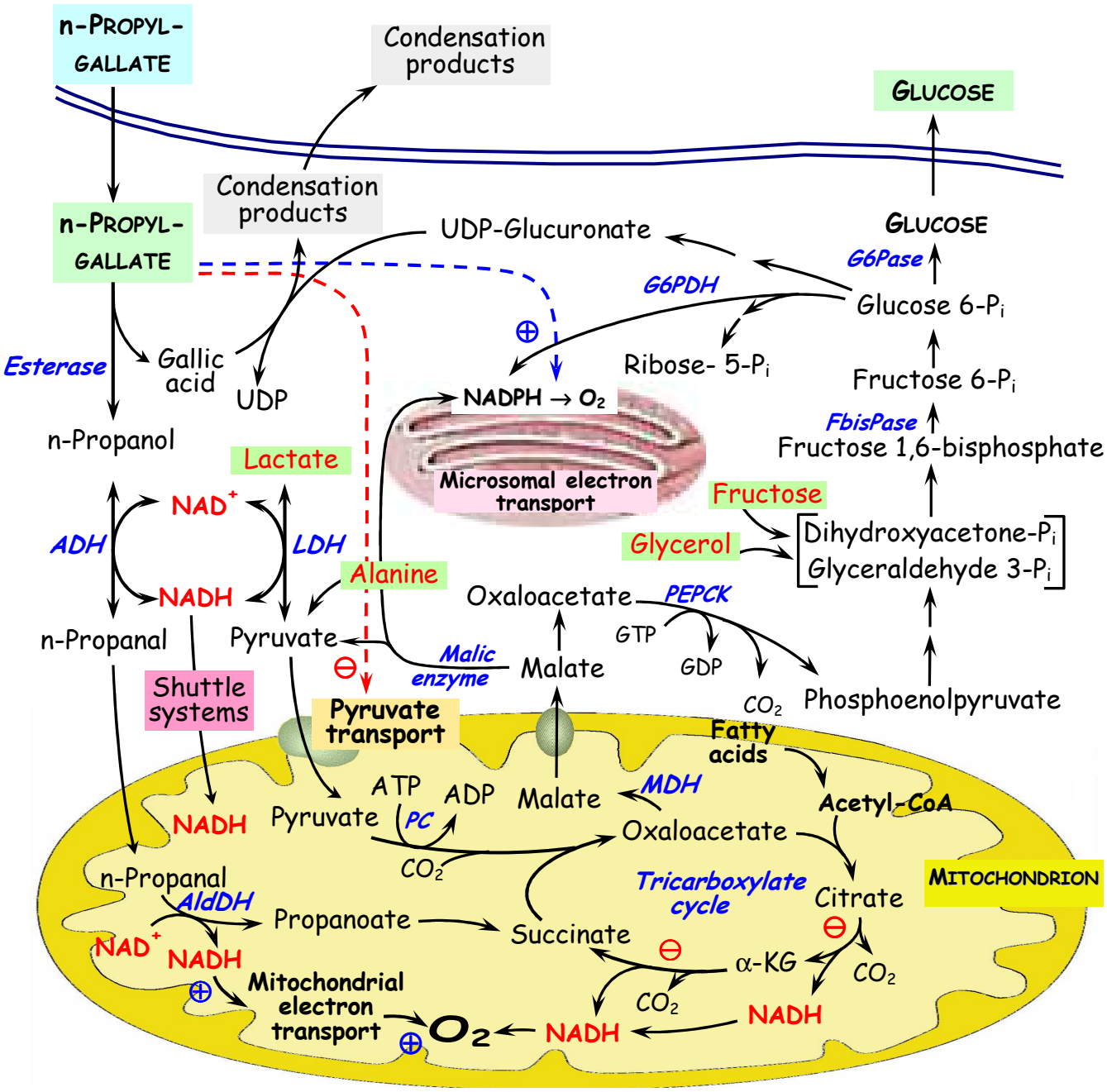

Figure 9**. Schematic representation of the main sites of action of n-propyl gallate in the liver cells.** Reactions and pathways are generally represented in abbreviated form. The symbol ⊕ means stimulation; ⊖ inhibition. The arrows indicate the predominant metabolic flux direction under the specific experimental conditions. Abbreviations: FbisPase, fructose 1,6-bisphosphatase; G6Pase, glucose 6-phosphatase; α-KG, α-ketoglutarate; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase; MDH, malate dehydrogenase; PC, pyruvate carboxylase; PEPCK, phosphoenolpyruvate carboxykinase; G6PDH, glucose 6-phosphate dehydrogenase.

The action of n-propyl gallate on glucose output and related parameters under glycogenolytic conditions in the rat liver

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Abstract

Based on a previous report that n-propyl gallate inhibits gluconeogenesis and stimulates oxygen uptake in the perfused rat liver, the present work presents measurements about the action of this compound on parameters derived from glycogen catabolism. Livers of fed rats were perfused in an open system using substrate-free perfusion fluid. n-Propyl gallate was infused at concentrations in the range between 50 and 400 μM. Glucose and lactate output under glycogenolytic conditions in livers from fed rats were increased by n-propyl gallate. Both effects were relatively mild and unstable under some conditions. In the passage from 200 μM to 400 μM n-propyl gallate the action on glucose release was further enhanced from 17% to 35% stimulation. n-Propanol, a major transformation product of n-propyl gallate, at the concentration of 200 μM, also produced small increases in glucose release. The relatively modest actions of n-propyl gallate on glucose and lactate output under glycogenolytic conditions are likely to have several causes linked to its inhibitory action on pyruvate transport into the mitochondria and to its transformation reactions. The latter include mainly the generation of n-propanol. Only at the concentration of 400 μM impairment of the mitochondrial energy metabolism could be playing a significant role. It remains to be investigated if other phenomena linked to the interactions of n-propyl gallate with the liver cells, such as the need for NADPH for microsomal electron transport stimulation and the need for UDP-glucuronate for glucuronidation reactions are also able to contribute for glycogenolysis stimulation.

Introduction

n-Propyl gallate (n-propyl 3,4,5-trihydroxybenzoate) is an ester formed by the condensation of gallic acid with n-propanol. It is an antioxidant and widely used in industrial products to prevent oxidation (Nakagawa et al., 1996). It also occurs in many products of plant origin as in green tea, for example (Galati et al., 2006). There are several studies in which the action of high concentrations of n-propyl gallate were investigated. It was found, for example, that n-propyl gallate, at the concentrations of 1 to 2 mM, causes cellular ATP depletion and cell death (Nakagawa et al., 1996a,b). The authors of this study concluded that the mitochondria are an important target of the toxic action of n-propyl gallate. In fact, it was possible to demonstrate that n-propyl gallate is able to reduce the respiratory control index in isolated liver mitochondria, evidence for an uncoupling action which could explain the cellular ATP depletion and cell death (Nakagawa et al., 1996a,b). An action on mitochondrial energy metabolism has been reiterated recently in a study in which the actions of several alkyl esters were investigated in mouse hepatocytes, mouse sarcoma 786A and mouse mammary carcinoma TA3 cell lines and its multiresistant variant TA3-MTX-R (Frey et al., 2007).

The concentrations of n-propyl gallate used in the experiments with intact cell systems, between 1 and 2 mM and which caused ATP depletion, are quite high (Nakagawa et al., 1996a,b ; Frey et al., 2007). Recent work of our laboratory has demonstrated that n-propyl gallate is already active on the liver metabolism at much lower concentrations without, however, depleting cellular ATP (Eler et al., 2009). The main effects were inhibition of glucose output (half-maximal inhibition of lactate gluconeogenesis at a concentration of 86.4 μM), stimulation of oxygen uptake (half-maximal stimulation at 42.8 μM) and inhibition of the tricarboxylic acid cycle. Inhibition of glucose output by n-propyl gallate up to 200 μM seems to be the consequence of a complex set of alterations which includes: a) impairment of gluconeogenesis in consequence of an inhibition of pyruvate transport across the mitochondrial membrane, possibly the most important alteration; b) deviation of a fraction of glucose 6-phosphate into the pentose-monophosphate shunt for the production of reducing equivalents in the form of NADPH to be used in the n-propyl gallate-stimulated microsomal electron transport; c) deviation of a fraction of glucose 6-phosphate for glucuronida-tion reactions; d) inhibition of gluconeogenesis by deviating cytosolic malate

(in equilibrium with oxaloacetate) for the production of NADPH to be used in the microsomal electron transport; e) gluconeogenesis inhibition by n-propanol, produced intracellularly from n-propyl gallate. n-Propanol oxidation is probably the main responsible for the increased mitochondrial respiration caused by n-propyl gallate, which is simultaneous to the inhibitory action on the tricarboxylic acid cycle.

All the actions listed above must undoubtly change the concentrations of many intermediate metabolites and metabolic regulators in the liver cell. Such changes usually affect the net fluxes of metabolic pathways. Inhibition of pyruvate transport into the mitochondria is also one of the steps that leads to fatty acid and cholesterol synthesis from glucose, which is diminished by inhibitors of monocarboxylate transport such as α-cyano-4-hydroxycinnamate and derivatives (Halestrap and Denton, 1975). *p*-Coumaric acid, another inhibitor of mitochondrial pyruvate transport, increases, though to a small extent, glucose and lactate output in the perfused liver under glycogenolytic conditions (Lima et al., 2006). This observation raises the question if a similar action can also be exerted by n-propyl gallate. In favour of this hypothesis it must be added that the transformation of n-propyl gallate generates n-propanol which is further metabolized (Eler et al., 2009). Since the transformation of ethanol also generates a situation which leads to increased glucose output under glycogenolytic conditions, a similar action of n-propanol can be expected (Lopez et al., 2004). And finally, although concentrations of n-propyl gallate up to 200 μM seem not to inhibit the mitochondrial energy metabolism in the perfused liver, it is also reasonable to expect that higher concentrations are perfectly able to exert such an action which also stimulates glycogenolysis for glycolytic purposes (Scholz and Bücher, 1965; Constantin et al., 1995; Acco et al., 2004). For all these reasons, in the present work, the action of n-propyl gallate on glycogen catabolism was investigated using various concentrations ranging from 50 to 400 μM. At this latter concentration one can already expect effects on energy metabolism in the intact cell if one takes into account experiments with isolated mitochondria. Experiments in the lower concentration range are, however, more important because they are more likely to occur in vivo.

Material and methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. *n*-Propyl gallate, gallic acid and all enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St. Louis, MO,USA). All other chemicals were from the best available grade (98–99.8% purity).

Animals

Male albino rats (Wistar), weighing 180–220 g, were fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil). All experiments were done in accordance with the internationally accepted recommendations in the care and use of animals.

Liver Perfusion

For the surgical procedure, the rats were anesthetized by intraperitoneal injection of thiopental (50 mg/kg). Hemoglobin-free, nonrecirculating perfusion was performed (Scholz and Bücher, 1965). After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 ^{o}C. The flow, provided by a peristaltic pump, was between 30 and 33 mL/min.

Analytics

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents. The following compounds were assayed by means of standard enzymatic procedures: glucose, lactate and pyruvate (Bergmeyer, 1974). The oxygen concentration in the out-flowing perfusate was monitored continuously, employing a Teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate (Scholz and Bücher, 1965). Metabolic rates were calculated from input-output differences and the total flow rates and were referred to the wet weight of the liver.

Treatment of data

The statistical significance of the differences between parameters obtained in the liver perfusion experiments was evaluated by means of Student's paired t test. The results are discussed in the text using p values; $p \leq 0.05$ was the criterion of significance.

Results

Several experiments were done in which n-propyl gallate in the range of 50 to 400 μM ml was infused into the portal vein during 20 minutes. Livers from fed rats were perfused with substrate-free perfusion fluid, in an open system. Under these conditions, the livers release glucose, lactate and pyruvate as a result of glycogen degradation (Scholz and Bücher, 1965; Bazotte et al., 1990). Figure 1 shows the time course of the experiments in which 400 μM n-propyl gallate was infused. Oxygen uptake and glucose release were both stimulated and remained elevated during the whole n-propyl gallate infusion time. Upon cessation of the infusion both variables tended to return to values close to those before initiation of the n-propyl gallate infusion (basal rates). Lactate production also increased shortly after initiation of n-propyl gallate infusion but the increase was not stable. After 14 minutes perfusion time (4 minutes after initiation of the n-propyl gallate infusion) the lactate production declined and reached values slightly below the basal rates. However, upon cessation of the infusion an additional decrease was observed. Pyruvate production declined only slightly.

Experiments like those shown in Figure 1 were repeated with four additional n-propyl gallate concentrations. The results are summarized in Figure 2 in terms of the changes above or below the basal rates. As shown by Figure 2 oxygen uptake stimulation was a saturable function of the portal n-propyl gallate concentration, confirming, thus, observations of our previous work (Eler et al., 2009). Lactate production and glucose release are more complex functions of the n-propyl gallate concentration. The former was actually inhibited at low n-propyl gallate concentrations (50 μM); stimulation took place at the concentrations of 200 and 400 μM. At 200 μM n-propyl gallate the stimulation of lactate production was stable (not shown). At 400 μM, however, it was unstable as already mentioned above (Figure 1) and the change represented in Figure 2 corresponds to the peak value. Glucose release stimulation, on the other hand, seemed to be saturated with 100 or 200 μM n-propyl gallate with approximately 17% stimulation, but there was another increase when the concentration was raised from 200 to 400 μM, reaching approximately 35% stimulation. The slight decrease in pyruvate production (maximally 20%), which is not clearly evident in Figure 1 because of the scale that was used, revealed to be statistically significant for the 200 and 400 μM concentrations. The decreased pyruvate production, when combined with the stimulated lactate production, means higher

lactate to pyruvate ratios. This, in turn, means also higher cytosolic NADH/NAD^+ ratios due to the lactate dehydrogenase equilibrium (Sies, 1982).

n-Propanol is the first product which results from n-propyl gallate transformation (Nakagawa et al., 1996b; Eler et al., 2009). In the preceding work we showed that 200 μM n-propanol stimulates oxygen consumption during substrate-free perfusion and inhibits lactate gluconeogenesis. For this reason, experiments were done in order to verify if some of the effects caused by n-propyl gallate could be due, partly at least, to the subsequent metabolic transformation of n-propanol. The mean values of these experiments are shown in Figure 3, in which a relatively low n-propanol concentration was used, namely 200 μM. n-Propanol produced a clear increment in oxygen uptake 0.33±0.05 ($p < 0.001$), an observation that confirms our previous report (Eler et al., 2009). Besides this, there were also significant changes in glucose release and pyruvate production. The mean increase in glucose release was 0.23±0.05 μmol min^{-1} g^{-1} ($p = 0.011$) whereas the mean decrease in pyruvate production was equal to −0.08±0.03 μmol min^{-1} g^{-1} ($p = 0.039$). There was also a tendency toward increased rates of lactate production, however, without statistical significance.

Discussion

This results of this work show that n-propyl gallate is able to stimulate glycogenolysis in the perfused rat liver at concentrations that are strongly inhibitory for gluconeogenesis. For example, n-propyl gallate 200 μM already inhibits alanine gluconeogenesis by 99% and lactate gluconeogenesis by 80% (Eler et al., 2009). However, the glycogenolytic action of n-propyl gallate at these concentrations (17 and 35% stimulation of glucose output at 200 and 400 μM, respectively) was relatively modest when compared to the action of other drugs and hormones. For example, 10 nM vasopressin is able to stimulate glucose output due to glycogenolysis by 277%. Antiinflammatory drugs acting on energy metabolism, such as diclofenac (0.5 mM) and niflumic acid (50 μM), are able to increase glucose output by 178 and 90%, respectively (Nascimento et al., 1993). Hormones like vasopressin and glucagon exert their effects by stimulating the production of secondary messengers which act specifically on the glycogen phosphorylase. Drugs like diclofenac, niflumic and many others stimulate glycogenolysis by virtue ot their negative action on ATP production in the mitochondrial respiratory chain leading to decreased cellular ATP and increased cellular AMP concentrations, the latter being apparently the main responsible for glycogenolysis stimulation via glycogen phosphorylase b (Baron et al., 1989). As shown by our previous work (Eler et al., 2009), however, n-propyl gallate up to 200 μM affects only minimally the cellular ATP content (−5%) and does not affect the AMP concentration. Consequently, its glycogenolytic action at concentrations up to 200 μM must have other causes than energy metabolism inhibition.

It has already been mentioned in the Introduction that *p*-coumaric acid, another inhibitor of mitochondrial pyruvate transport, also increases glycogen catabolism though to a very small extent (Lima et al., 2004). However, the action of this compound on pyruvate transport is incomplete (maximally 65%), whereas the action of n-propyl gallate is complete (i.e., 100% inhibition can eventually be achieved by raising the concentration). So, if one assumes that inhibition of mitochondrial pyruvate transport of not more than 65% is able to generate a regulatory situation that leads to increased glycogen degradation, one should expect a more pronounced effect when inhibition approaches 90 to 100%. Furthermore, the intracellular appearance of n-propanol in consequence of n-propyl gallate transformation evidently contributes to glycogenolysis stimulation as shown by the specific experi-

ments in which this alcohol was infused. On the other hand, the increased rates of glucose and lactate output could also be partly reflecting the reduced intramitochondrial transformation of pyruvate. Inhibition of pyruvate transport into the mitochondria does not only inhibit its carboxylation to oxaloacetate, as shown by our previous work, but it also inhibits its transformation into acetyl-CoA via pyruvate dehydrogenase (Scholz et al., 1978). The excess lactate output caused by n-propyl gallate could be reflecting in part this phenomenon because of the equilibrium of the lactate dehydrogenase reaction (Sies, 1982) which in the presence of n-propanol is shifted in the direction of a more reduced state due to the higher NADH/NAD^+ ratios. The latter phenomenon is the usual consequence of alcohol transformation which carries an excess of reducìng equivalents into the respiratory chain. Oxidation of this excess stimulates oxygen uptake (Figure 3) and inhibits the tricarboxylic acid cycle (Lopez et al., 2004). In this respect it is worth to emphasize that inhibition of the tricarboxylic acid cycle is one of the observed effects of n-propyl gallate (Eler et al., 2009).

In conclusion, the relatively modest actions of n-propyl gallate on glucose and lactate output under glycogenolytic conditions are likely to have several causes linked to its inhibitory action on pyruvate transport into the mitochondria and to its transformation reactions. The latter include mainly the generation of n-propanol. Only at the concentration of 400 μM impairment of the mitochondrial energy metabolism could be playing a significant role. It remains to be investigated if other phenomena linked to the interactions of n-propyl gallate with the liver cells, such as the need for NADPH for microsomal electron transport stimulation (Eler et al., 2009) and the need for UDP-glucuronate for glucuronidation reactions (Bánhegyi et al., 1988; Nakagawa et al., 1996b) are also able to contribute for glucose output stimulation under glycogenolytic conditions.

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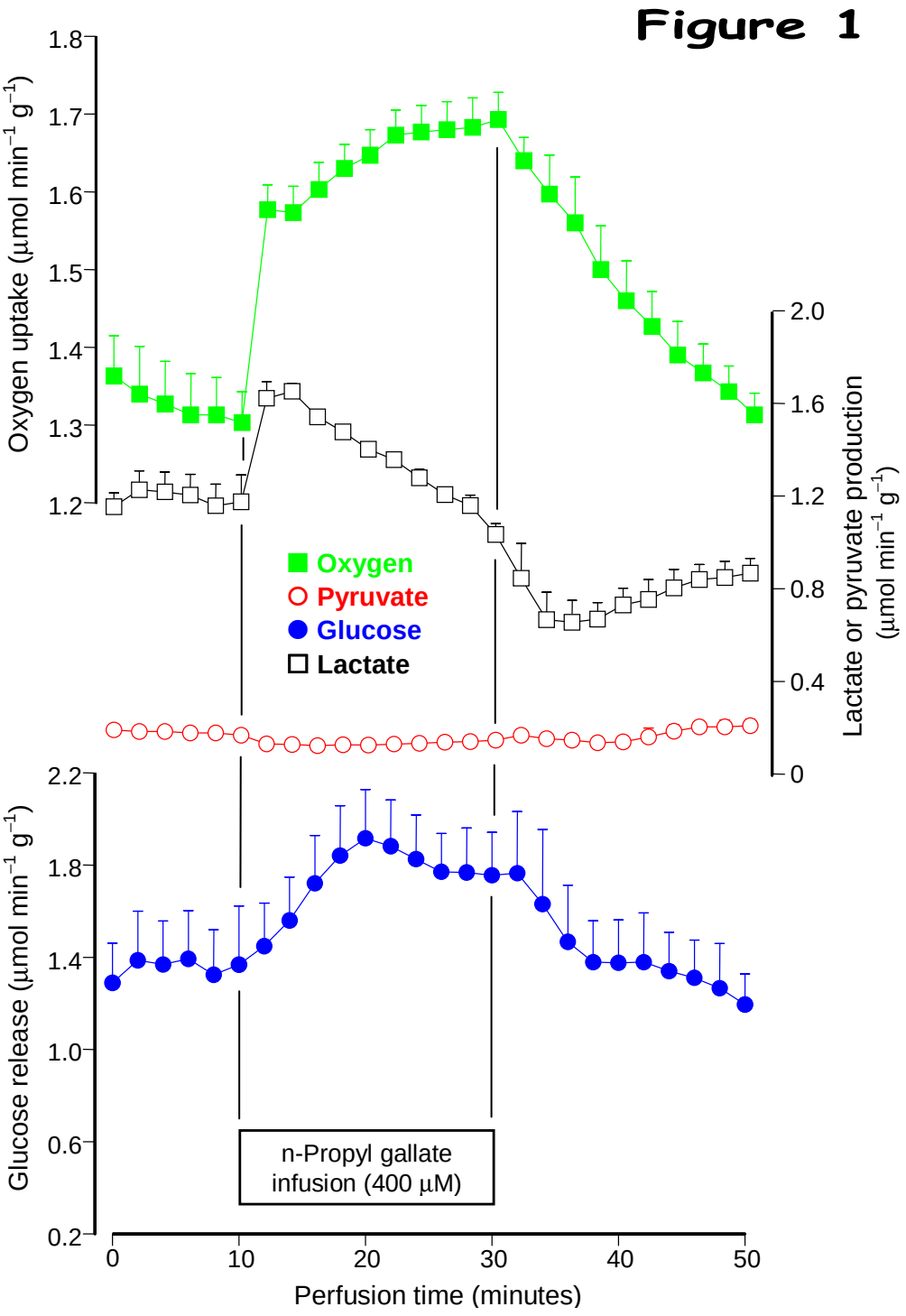

Figure 1**. Time course of the effects of 400 µM n-propyl gallate on metabolic fluxes derived from glycogen catabolism and on oxygen uptake in livers from fed rats.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in the Materials and Methods section. Samples of the effluent perfusate were withdrawn for glucose, lactate and pyruvate assay. Oxygen in the venous perfusate was monitored polarographically. The data represent the means (±SEM) of four liver perfusion experiments.

Figure 2

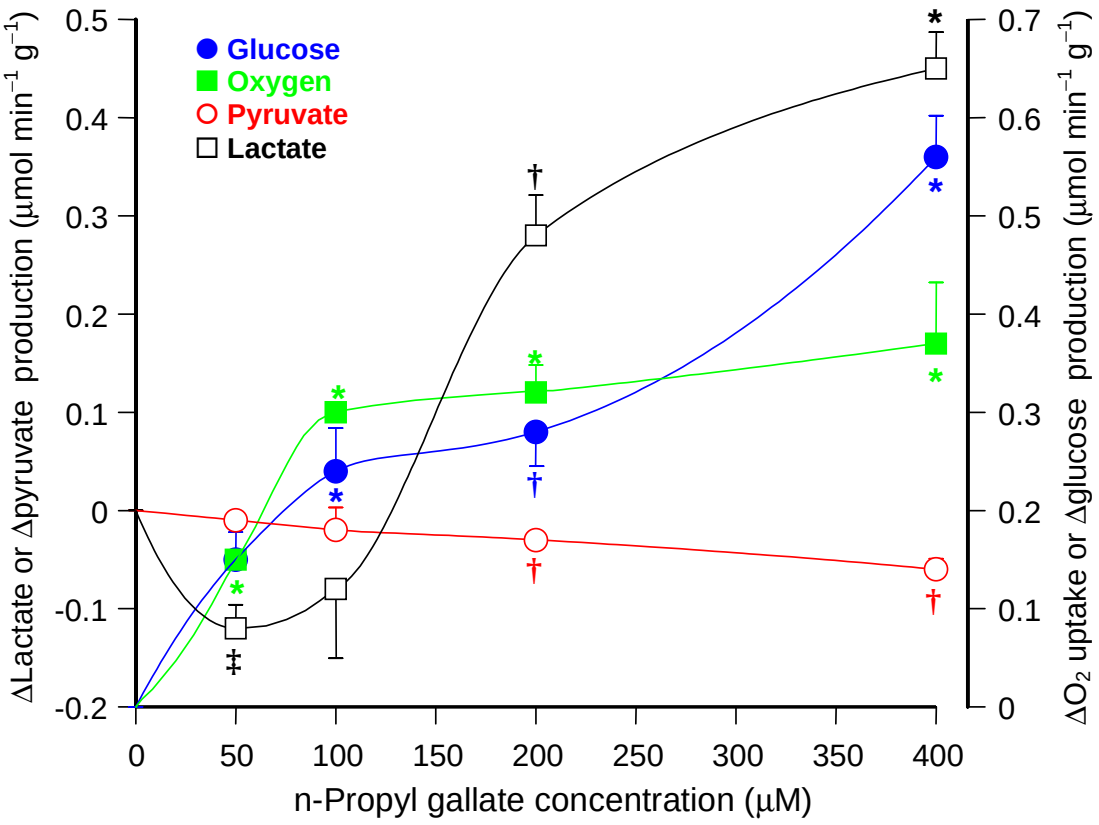

Figure 2**. Concentration dependences of the changes caused by n-propyl gallate glycogen catabolism and oxygen uptake in the perfused liver of fed rats.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) according to the protocol illustrated by Figure 1 using various n-propyl gallate concentrations. The changes caused by n-propyl gallate were evaluated and represented against the n-propyl gallate concentration. These changes were calculated as the difference between the basal rates (absence of n-propyl gallate) and the maximal (or minimal) rate in the presence of n-propyl gallate. The data points represent the means ± mean standard errors of 3-4 liver perfusion experiments. Statistical significance was evaluated by applying Student's paired t test. The results are indicated by the symbols *****, † and ‡ meaning, respectively, $p \leq 0.01$, $p \leq 0.02$ and $p \leq 0.05$.

Figure 3

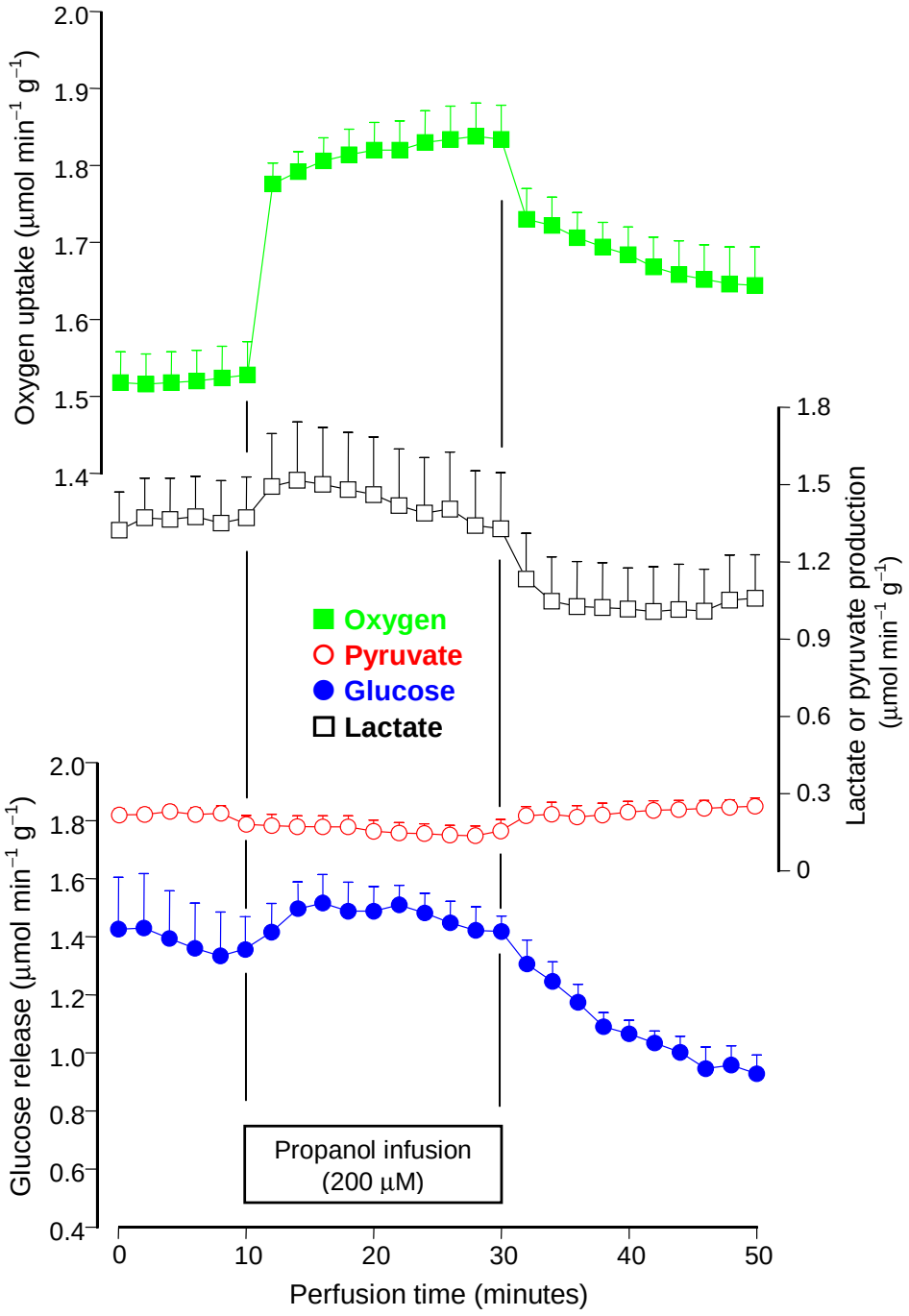

Figure 3**. Time course of the effects of 200 µM n-propanol on metabolic fluxes derived from glycogen catabolism and on oxygen uptake in livers from fed rats.** Livers were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4) as described in the Materials and Methods section. Samples of the effluent perfusate were withdrawn for glucose, lactate and pyruvate assay. Oxygen in the venous perfusate was monitored polarographically. The data represent the means (±SEM) of five liver perfusion experiments.

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