

Efeitos metabólicos da carbenoxolona no fígado de rato

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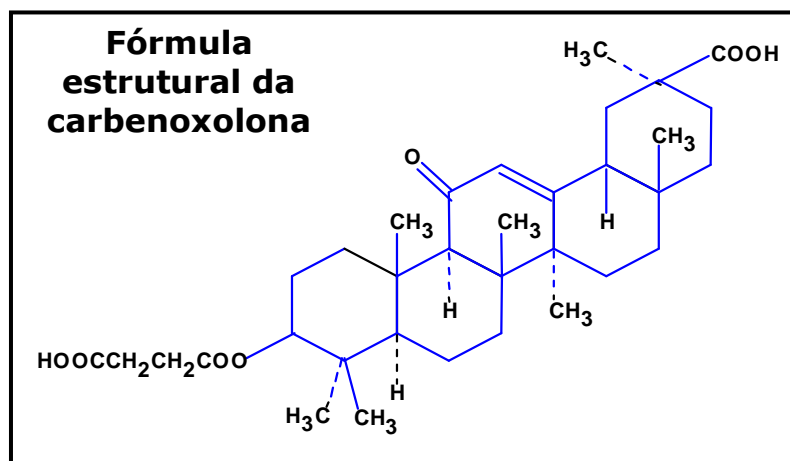
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Resumo

INTRODUÇÃO E OBJETIVOS — A carbenoxolona é um derivado do ácido glicirretínico, o princípio ativo do alcaçuz (*Glycyrrhiza glabra*), uma raiz medicinal.



As propriedades farmacológicas atribuídas à carbenoxolona são relacionadas às suas ações inibitórias sobre a 11β -hidroxiesteróide desidrogenase e canais *gap junctions*. Foi demonstrado que o bloqueio da comunicação intercelular através de *gap junctions* (gap junctional intercellular communication) pela carbenoxolona reduz a eficiência da diapedese de células tumorais, um processo envolvido na migração destas células malignas (metástase). Além de bloquear a comunicação intercelular, o ácido glicirretínico e seus derivados exibem atividades antiinflamatória, antiulcerogênica e antiviral. Estudos recentes têm mostrado que a carbenoxolona também induz swelling e colapso do potencial de membrana em mitocôndrias de fígado. Estes efeitos foram relacionados à geração de peróxido de hidrogênio e à indução da transição de permeabilidade (*mitochondrial permeability transition*). Isto indica possíveis ações toxicológicas da carbenoxolona sobre as mitocôndrias, o que poderia dar início a apoptose. Considerando-se que impedimentos na capacidade bioenergética de mitocôndrias poderiam causar alterações metabólicas no fígado, o presente trabalho foi planejado para investigar uma possível ação da carbenoxolona sobre o metabolismo energético celular do fígado.

MÉTODOS — Ratos machos Wistar, pesando 180 a 220 g foram usados em todos os experimentos. Nos experimentos de perfusão, os fígados isolados de ratos alimentados ou em jejum de 24 horas foram perfundidos no modo não-recirculante. Glicose, lactato e piruvato liberados no perfusado foram medidos através de procedimentos enzimáticos padronizados. Os fluxos metabólicos foram calculados a partir das diferenças porto-venosas e o fluxo através do fígado e referidos ao peso fresco do órgão. Mitocôndrias de fígados de ratos foram isoladas por centrifugação diferencial em meio de manitol e sacarose para os testes polarográficos e enzimáticos. O consumo de oxigênio de mitocôndrias intactas foi medido polarograficamente adicionando-se os seguintes substratos ao meio de incubação: β -hidroxibutirato, succinato e ADP. Mitocôndrias rompidas

por congelamento-descongelamento foram usadas para o ensaio da atividade da ATPase.

RESULTADOS — Os principais resultados foram os seguintes:

1) A transformação do lactato em glicose (neoglicogênese) nos fígados de ratos em jejum foi inibida pela carbenoxolona na faixa de concentração entre 25 e 200 μM . O grau de inibição foi de 97% na concentração de 200 μM . O consumo de oxigênio estimulado pelo lactato também foi inibido nesta faixa de concentração.

2) A neoglicogênese a partir de frutose e o estímulo do consumo de oxigênio devido à infusão deste substrato foram também inibidos. Uma inibição máxima de 94% na produção de glicose foi obtida com carbenoxolona 200 μM .

3) Nos fígados de ratos alimentados a carbenoxolona aumentou o consumo de oxigênio, a glicólise e a glicogenólise. A degradação do glicogênio foi aumentada em 158% e 213%, respectivamente, pela carbenoxolona 200 e 300 μM . A atividade glicolítica foi estimulada em 269% com carbenoxolona 300 μM . O consumo de oxigênio foi estimulado pela carbenoxolona nas concentrações de 200 e 300 μM .

4) A carbenoxolona 200 μM aumentou em até 26 vezes a liberação de lactato desidrogenase no perfusado durante um período de infusão de 30 minutos.

5) Os níveis celulares de ATP foram diminuídos pela carbenoxolona, enquanto que os de AMP foram aumentados. O conteúdo total de nucleotídeos foi também diminuído.

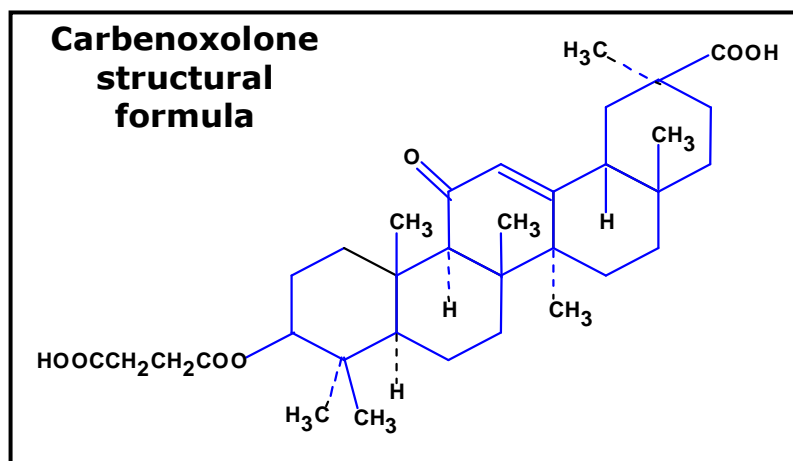
6) Em mitocôndrias isoladas, a carbenoxolona aumentou a respiração no estado IV e a respiração dependente apenas das oxidações do succinato e do β -hidroxibutirato, mas diminuiu a respiração no estado III e o coeficiente de controle respiratório (RC).

7) A carbenoxolona estimulou a atividade ATPásica de mitocôndrias intactas e inibiu a de mitocôndrias desacopladas. A atividade ATPásica de mitocôndrias rompidas por congelamento não foi alterada.

DISCUSSÃO E CONCLUSÕES — Os dados permitem concluir que a carbenoxolona afeta várias vias metabólicas no fígado, possivelmente reduzindo a eficiência da transdução de energia nas mitocôndrias através de uma atividade desacopladora. Esta conclusão tem como base as observações de que o nível de ATP celular e o conteúdo total de nucleotídeos foram diminuídos. Além disto, a carbenoxolona ativou a glicogenólise e a glicólise e inibiu a neoglicogênese, uma combinação de fenômenos esperados para a formação diminuída de ATP. A maioria das alterações metabólicas produzidas pela carbenoxolona foi similar àquela causada por inibidores clássicos da fosforilação oxidativa. A ação desacopladora foi inferida de seus efeitos sobre a respiração mitocondrial e atividade ATPásica, os quais foram, a estimulação do estado IV da respiração, o decréscimo do controle respiratório e o aumento da hidrólise de ATP em mitocôndrias acopladas intactas. A ação inibitória da carbenoxolona sobre o metabolismo energético mitocondrial poderia contribuir para a indução da transição de permeabilidade (MPT) e para o desencadeamento da apoptose. Os resultados do presente estudo podem explicar, em parte ao menos, a ação hepatotóxica da carbenoxolona encontrada numa avaliação clínica.

Abstract

INTRODUCTION AND AIMS — Carbenoxolone is a derivative of glycyrrhetic acid, the active principle of licorice (*Glycyrrhiza glabra*), a medicinal root.



The pharmacological properties attributed to carbenoxolone are related to its inhibitory actions on the 11β -hydroxysteroid dehydrogenase and gap junction channels. It was observed that carbenoxolone, by blocking the gap junctional intercellular communications (GJIC) reduces the efficiency of tumor cell diapedesis, a process involved in the migration of these malignant cells (metastasis). In addition to blocking the gap junctional intercellular communications, glycyrrhetic acid and its derivatives exhibit antiinflammatory, antiulcerous and antiviral activities. Recent studies have shown that carbenoxolone also induces swelling and membrane potential collapse in mitochondria. These effects were related to hydrogen peroxide generation and mitochondrial permeability transition (MPT) induction, indicating possible toxicological actions of carbenoxolone at the mitochondrial level, which could trigger the apoptotic pathway. Since impairment of the bioenergetic capacity of mitochondria could cause metabolic changes in the liver, the present work was undertaken to investigate the action of carbenoxolone on liver cell energy metabolism.

METHODS — Male Wistar rats weighing 180 to 220 g were used in all experiments. In the perfusion experiments, the livers isolated from fed or 24 hours fasted rats were perfused in the non-recirculating mode. Glucose, lactate and pyruvate released into the perfusate were measured by standard enzymatic procedures. Metabolic rates were calculated from input-output differences and the flow through the liver and were referred to the wet weight of the organ. Rat liver mitochondria were isolated by differential centrifugation in a mannitol-sucrose medium for polarographic and enzymatic assays. Oxygen consumption by intact mitochondria was measured polarographically adding the following substrates to the incubation medium: β -hydroxybutyrate, succinate and ADP. Freeze-thawing disrupted mitochondria were used for assaying ATPase activity.

RESULTS — The main results were the following:

1) The transformation of lactate into glucose (gluconeogenesis) in livers from fasted rats was inhibited by carbenoxolone in the concentration range between 25 and 200 μM . The inhibition degree was 97% at the concentration of 200 μM . Oxygen consumption stimulated by lactate was also inhibited in the same concentration range.

2) Gluconeogenesis from fructose and oxygen uptake stimulated by the substrate infusion were also inhibited. The maximal inhibition of 94% in glucose production was obtained with 200 μM carbenoxolone.

3) In livers from fed rats carbenoxolone increased oxygen uptake, glycolysis and glycogenolysis. Glycogen breakdown was increased by 158% and 213%, respectively, with 200 and 300 μM carbenoxolone. The glycolytic activity was increased by 269% with 300 μM carbenoxolone. Oxygen consumption was stimulated by carbenoxolone at the concentrations of 200 and 300 μM .

4) The lactate dehydrogenase release into the perfusate was increased up to 26-fold during a 30 minutes infusion period of 200 μM carbenoxolone.

5) The cellular ATP levels were decreased by carbenoxolone, whereas the AMP levels were increased. The total adenine nucleotide content was also decreased.

6) In isolated mitochondria, carbenoxolone increased state IV respiration and respiration dependent solely on succinate and β -hydroxybutyrate oxidation. However, it decreased state III respiration and diminished the respiratory control ratio.

7) Carbenoxolone stimulated the ATPase activity of intact mitochondria and inhibited the ATPase activity of uncoupled mitochondria. The ATPase activity of freeze-thawing disrupted mitochondria was not altered.

DISCUSSION AND CONCLUSION — The data allow us to conclude that carbenoxolone affects several metabolic pathways in the liver possibly reducing the efficiency of energy transduction in the mitochondria by acting as an uncoupler. This conclusion was based on the observations that the cellular ATP and the total nucleotide contents were decreased in both perfused livers from fed and fasted rats. In addition to this, carbenoxolone activated glycogenolysis and glycolysis and inhibited gluconeogenesis, which is an expected combination of phenomena for decreased mitochondrial ATP formation. Most of the metabolic alterations produced by carbenoxolone in perfused rat livers and isolated mitochondria were similar to those ones caused by classic inhibitors of oxidative phosphorylation. The uncoupling action was inferred from its effects on mitochondrial respiration and ATPase activity, namely stimulation of state IV respiration, decrease of the respiratory control ratio, and increase of ATP hydrolysis in intact coupled mitochondria. The inhibitory action of carbenoxolone on mitochondrial energy metabolism could contribute to induce the mitochondrial permeability transition and to trigger the apoptotic pathway. The results of the present study can explain, partly at least, the in vivo hepatotoxic actions of carbenoxolone that were found in a previous clinical evaluation.

Metabolic effects of carbenoxolone in the rat liver

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Abstract

The action of carbenoxolone on hepatic energy metabolism was investigated in the perfused rat liver and isolated mitochondria. In perfused livers carbenoxolone (200-300 μM) increased oxygen consumption, glucose release and glycolysis from endogenous glycogen. Gluconeogenesis from lactate or fructose, an energy-dependent process, was inhibited. This effect was already evident at a concentration of 25 μM . The cellular ATP levels and the adenine nucleotide content were decreased by carbenoxolone, whereas the AMP levels were increased. In isolated mitochondria, carbenoxolone stimulated state IV respiration and decreased the respiratory coefficient with the substrates β -hydroxybutyrate and succinate. The ATPase of intact mitochondria was stimulated, the ATPase of uncoupled mitochondria was inhibited and the ATPase of disrupted mitochondria was not altered by carbenoxolone. These results indicate that carbenoxolone acts as an uncoupler of oxidative phosphorylation and, possibly, as an inhibitor of the ATP/ADP exchange system. The inhibitory action of carbenoxolone on mitochondrial energy metabolism could be contributing to induce the mitochondrial permeability transition (MPT), a key phenomenon in apoptosis. The results of the present study can explain, partly at least, the *in vivo* hepatotoxic actions of carbenoxolone that were found in a previous clinical evaluation.

1. Introduction

Carbenoxolone is the 3-hemisuccinate of glycyrrhetic acid, the active principle of licorice (*Glycyrrhiza glabra*), a medicinal root [1]. Most of the pharmacological properties attributed to carbenoxolone are related to its inhibitory actions on the 11β -hydroxysteroid dehydrogenase [2] and on gap junction channels [3]. It has been claimed that in vivo carbenoxolone exerts anticonvulsant, muscle relaxant and hypnotic effects, possibly by blocking gap junction channels [4]. A recent study [5] demonstrated that blocking of heterocellular gap junctional communication with carbenoxolone in co-cultures reduces diapedesis of connexin 43 expressing HBL 100 breast cancer, a process involved in the extravasion of malignant cells (metastasis). This finding suggests that there might be therapeutic advantages in designing drugs that inhibit vascular homocellular and heterocellular gap junctional communication as a means of reducing metastatic spread of tumor cells. In addition to blocking gap junctional communication, glycyrrhetic acid and its derivatives have been shown to exhibit antiinflammatory, antiulcerous and antiviral activities [6].

Recently it has been shown [7] that carbenoxolone induces swelling and membrane potential collapse when added at micromolar concentrations to liver mitochondria. These effects were related to hydrogen peroxide generation, increase in oxygen uptake and sulfhydryl and pyridine nucleotide oxidation, which could also trigger the apoptotic pathway, since the above events would result in the loss of cytochrome c. It was concluded [7] that in rats the pro-apoptotic effect of carbenoxolone is due not only to the inhibition of 11β -hydroxysteroid dehydrogenase, which enhances corticosterone availability in the cells, but also to induction of mitochondrial permeability transition indicating, thus, a potentially toxic action of carbenoxolone at the mitochondrial level. At the same time, however, since apoptosis is considered a safety mechanism activated by organisms in order to eliminate absorbed or damaged cells, the two pathways utilized by glycyrrhetic acid in inducing apoptosis could equally explain some apparently beneficial effects exhibited by licorice and its derivatives, namely the antiinflammatory, antiviral, and anticarcinogenic actions [8].

The data of these previous reports [7,8] are pointing, thus, in the direction of a possible action of carbenoxolone on the bioenergetic functions of mitochondria,

which could in turn cause toxic metabolic changes in the liver. Oral administration of carbenoxolone to patients during relatively long periods (30 days) produced high plasma levels of alanine and aspartate aminotransferases and abnormal bromosulfophthalein clearance, observations that have been interpreted as evidence of liver damage [9]. Within this context, the aim of the present study was to evaluate the possible action of carbenoxolone on the cellular energy metabolism and to identify the target and the mechanism of its action on the liver mitochondria. For this purpose we have examined the influence of carbenoxolone on respiration and carbohydrate metabolism in perfused rat livers and on the respiratory activity and some enzymatic activities in isolated mitochondria.

2. Materials and methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Several peristaltic pumps used in the experiments were a gift of Dr. Roland Scholz of the Institute for Physiological Chemistry of the University of Munich, Germany. Carbenoxolone and all enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St. Louis, US). 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 some experimental protocols the rats were starved for 24 hours before the surgical removal of the liver.

Liver perfusion

For the surgical procedure the rats were anesthetized by intraperitoneal injection of thiopental (50 mg/kg). Hemoglobin-free, non-recirculating perfusion was performed. The surgical technique was that one described by Scholz and Bücher [10]. 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°C. The flow, provided by a peristaltic pump, was between 30 and 32 ml/min.

Livers from animals in two different metabolic conditions were used: *ad libitum* fed rats and 24-hours fasted rats. Substrate-free perfused livers from fed rats respire mainly at the expense of endogenous fatty acids, but at the same time they exhibit extensive glycogenolytic and glycolytic activity [10]. Livers from fasted rats also respire at the expense of endogenous fatty acids, but their glycogen levels are very low [11]. This is a suitable condition for measuring gluconeogenesis from exogenous substrates without interference by glycogen catabolism. In the present work two different substrates were infused: lactate and fructose.

Analytical

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analysed for their metabolite contents. The following compounds were assayed by means of standard enzymatic procedures: glucose [12], lactate [13] and pyruvate [14]. 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 [10]. Metabolic rates were calculated from input-output differences and the total flow rates and were referred to the wet weight of the liver.

The hepatic contents of AMP, ADP and ATP 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 assayed by means of standard enzymatic procedures [15,16].

The activity of lactate dehydrogenase in the effluent perfusate was assayed by measuring the initial rate of NADH oxidation in a medium containing 4 mM pyruvate, 0.15 mM NADH and 15 mM phosphate buffer (pH 7.4) [14,17].

Mitochondria isolation and measurement of the respiratory activity

Fed rats, weighing between 180 and 220 g, were decapitated and their livers removed immediately and cut into small pieces. These fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 1.0 mM Tris-HCl (pH 7.4), 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (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 [18] and suspended in the same medium, which was kept at 0-4°C.

Oxygen uptake by isolated mitochondria was measured polarographically using a teflon shielded platinum electrode [18,19]. Mitochondria (0.85 ± 0.35 mg protein/ml) were incubated in the closed oxygraph chamber in a medium (2.0 ml) containing 0.25 M mannitol, 5 mM sodium phosphate, 10 mM KCl, 0.2 mM EDTA, 25 mg% fatty acid-free bovine serum albumin, 10 mM Tris-HCl (pH 7.4) and two different substrates in addition to various carbenoxolone concentrations

in the range between 20 and 200 μM . The substrates were succinate (10 mM) and β -hydroxybutyrate (10 mM). ADP, for a final concentration of 0.125 mM, was added at appropriate times. Rates of oxygen uptake were computed from the slopes of the recorder tracings and expressed as nmol per minute per mg protein. The respiratory control ratio (RC) and the ADP/O ratio were calculated according to Chance and Williams [20]. Protein content of the mitochondrial suspensions was measured by means of the method described by Lowry et al. [21], using the Folin-phenol reagent and bovine-serum albumin as a standard.

ATPase activity

The mitochondrial ATPase activity was measured in intact (coupled and uncoupled) and in freeze-thawing disrupted mitochondria [22]. When intact mitochondria were used as enzyme source, the reaction medium contained: 0.2 M sucrose, 10 mM TRIS-HCl (pH 7.4), 50 mM KCl, 0.2 mM EGTA and, when required, 0.2 mM 2,4-dinitrophenol (uncoupled mitochondria). When disrupted mitochondria were incubated, the medium contained 20 mM TRIS-HCl (pH 7.4). The reaction was started by the addition of 5 mM ATP and stopped, after 20 min of incubation at 37°C, by the addition of ice-cold 5% trichloroacetic acid. Phosphate was measured as described by Fiske and Subbarow [23].

Treatment of data

The statistical significance of the differences between parameters obtained in the liver perfusion experiments was evaluated by means of Student's **t**-test or by Newman-Keuls test after submitting the data to variance analysis according to context. The results are mentioned in the text as the **p** values; **p** < 0.05 was the criterion of significance.

3. Results

Effects of carbenoxolone on oxygen consumption and gluconeogenesis in livers of fasted rats

In the first experiments the action of carbenoxolone on oxygen consumption and gluconeogenesis from lactate was investigated in perfused rat livers in order to verify the effects of carbenoxolone on an energy-dependent biosynthetic process. Figure 1 shows the time courses of glucose production and oxygen uptake obtained in a series of experiments in which 2 mM lactate and 100 μM carbenoxolone were infused in livers of 24-hours fasted rats. Both glucose production and oxygen uptake increased progressively upon introduction of lactate, tending to stabilize at 30 minutes infusion time. The introduction of 100 μM carbenoxolone produced progressive decreases in both gluconeogenesis and oxygen uptake. It has been amply demonstrated that the perfused rat liver maintains a stable gluconeogenic activity for at least two hours [24]. Experiments like those shown in Figure 1 were repeated with several carbenoxolone concentrations and the results are summarized in Figure 2. The control values (absence of carbenoxolone) correspond to the rates found in the presence of lactate just before the onset of carbenoxolone infusion (30 minutes perfusion time) minus the basal rates (i.e., before the onset of lactate infusion). Rates in the presence of lactate + carbenoxolone were evaluated at 70 minutes perfusion time and also subtracted from the basal rates. Figure 2 reveals that both oxygen uptake and gluconeogenesis were a negative function of the carbenoxolone concentration. An inhibition of about 97% in glucose production was observed at the concentration of 200 μM ; 50% inhibition can be expected at a concentration of 53.1 μM , as computed by numerical interpolation. Oxygen uptake was also diminished progressively in the presence of carbenoxolone. The inhibition degree of the lactate stimulated respiration was 80% at 200 μM carbenoxolone; 50% inhibition can be expected at a concentration of 84 μM , as computed by numerical interpolation.

Experiments similar to those ones with lactate as the gluconeogenic precursor were also done with fructose at a concentration of 5 mM. It is well known that this substrate can also be transformed into glucose in the liver, but ramification of the fructose pathway at the enolase step can also lead to the production of

lactate and pyruvate [25]. The experimental protocol was that one illustrated by Figure 3. Both gluconeogenesis and oxygen uptake increased progressively upon fructose introduction, tending to stabilize at 30 minutes infusion time. The introduction of 100 μM carbenoxolone produced changes in all variables, but their kinetics was relatively complex. Oxygen uptake was rapidly inhibited with a minimum at 34 minutes perfusion time. This inhibition was followed by a partial recovery. Lactate production was stimulated with a maximum around 46 minutes perfusion time and a partial decline thereafter. Glucose production was inhibited with a minimum around 54 minutes perfusion time and a partial recovery during the rest of the carbenoxolone infusion time. Pyruvate production, finally, suffered an initial small inhibition, which was followed by a stable stimulation. The same pattern of response was obtained with 25, 50 and 200 μM carbenoxolone with the difference that, in most cases, the extent of the effects was a function of the concentration. The mean alterations in oxygen uptake and glucose production at the end of the carbenoxolone infusion (66 minutes perfusion time) are summarized in Figure 4. A maximal inhibition of 94% in glucose production was obtained with 200 μM carbenoxolone. The same carbenoxolone concentration produced a decrease of about 75% in the fructose stimulated oxygen consumption. For glucose production 50% inhibition can be expected at a concentration of 86.5 μM as computed by numerical interpolation.

Effects of carbenoxolone on glycogen catabolism and oxygen consumption in livers of fed rats

The action of carbenoxolone on glycogen catabolism and oxygen uptake in the liver of fed rats was examined in the concentration range between 50 and 300 μM . The drug was infused during 20 minutes. The following parameters were measured: glucose release, lactate and pyruvate production and oxygen consumption. Figure 5 shows the time courses of the changes caused by 200 μM carbenoxolone. The infusion of 200 μM carbenoxolone increased both glucose and lactate release. The drug also promoted increase in oxygen uptake and a small decrease in pyruvate production. These effects were reversible, i.e., they vanished upon cessation of the infusion but the reversion occurred slowly after a lag phase of approximately 5 minutes. Experiments like those shown in Figure 5 were repeated with several carbenoxolone concentrations and the mean results are summarized in Figure 6. Control values (absence of carbenoxolone)

correspond to the basal rates just before the onset of carbenoxolone infusion (10 minutes perfusion time). Rates of oxygen uptake, glycolysis ($[\text{lactate} + \text{pyruvate}]/2$) and glycogenolysis (glucose + glycolysis) in the presence of several carbenoxolone concentrations were evaluated at 30 minutes perfusion time. Figure 6 reveals that carbenoxolone was without action on these parameters at concentrations up to 100 μM . Glycogenolysis, however, was increased by 158% and 213%, respectively, with 200 and 300 μM carbenoxolone. For glycolysis the corresponding increments were equal to 178 and 269%, respectively; and, for oxygen uptake 24 and 17%, respectively.

Lactate dehydrogenase release in the perfused liver in the presence of carbenoxolone

According to a previous report, prolonged treatment of patients with carbenoxolone increases the plasma levels of lactate dehydrogenase and other enzymes [9]. Figure 7 shows the results of a series of experiments that were done in an attempt of reproducing this finding in the isolated perfused rat liver. Carbenoxolone (200 μM) was infused during 30 minutes in livers from fed rats and the activity of lactate dehydrogenase in the outflowing perfusate was measured. The basal release of lactate dehydrogenase was minimal. Carbenoxolone increased the lactate dehydrogenase release up to 26-fold (at 32 minutes perfusion time). After cessation of carbenoxolone infusion the lactate dehydrogenase levels in the outflowing perfusate experienced a progressive decrease.

Effects of carbenoxolone on isolated mitochondria

If the action of carbenoxolone on hepatic oxygen uptake is of mitochondrial origin it should be possible, in principle at least, to reproduce these effects in isolated mitochondria. For this purpose the influence of carbenoxolone on the respiration of isolated rat liver mitochondria was measured using two different substrates as electron donors. As revealed by Figure 8 (A and B) these substrates were succinate (FAD-dependent) and β -hydroxybutyrate (NAD^+ -dependent). The mitochondrial respiration driven by the oxidation of these substrates was measured in isolated rat liver mitochondria incubated in the absence of exogenous ADP (substrate respiration), presence of exogenous ADP

(state III respiration) and after the exhaustion of the exogenously added ADP (state IV respiration) in the absence and presence of several carbenoxolone concentrations.

Irrespective of the substrate that was used in the assays, carbenoxolone affected oxygen uptake of isolated mitochondria only at high concentrations. Figure 8A and 8B reveals that before ADP addition (substrate respiration) and after exhaustion of ADP (state IV respiration) carbenoxolone increased oxygen uptake in a dose-dependent manner with both substrates (succinate and β -hydroxybutyrate). In the presence of ADP (state III respiration) carbenoxolone caused inhibition of oxygen uptake at the concentrations of 160 and 200 μM . Furthermore, the respiratory control was almost abolished, as can be judged from the state IV respiration rates and from the respiratory control ratios (state IV/state III) in Table 1. The ADP/O ratios were also evaluated and listed in Table 1. They were not affected by carbenoxolone when succinate was the substrate. Significant effects were seen with β -hydroxybutyrate only at the highest carbenoxolone concentrations (160 and 200 μM).

Effects of carbenoxolone on the ATPase activity

The effects of carbenoxolone on the ATPase activity were measured in intact mitochondria either in the absence (coupled mitochondria) or in the presence of 2,4-dinitrophenol (uncoupled mitochondria) and in freeze-thawing disrupted mitochondria, as shown in Figure 9. The actions of carbenoxolone were different in each preparation. The ATPase activity of coupled mitochondria was increased over the whole concentration range of carbenoxolone. Numerical interpolation reveals that 100% stimulation can be expected at a carbenoxolone concentration of 21.8 μM . The ATPase activity of uncoupled mitochondria, on the other hand, was inhibited. This inhibition was significant within the concentration range of 160 to 200 μM . When disrupted mitochondria were used as the enzyme source, the ATPase activity was not significantly affected by carbenoxolone.

Effects of carbenoxolone on the hepatic adenine nucleotide contents

Inhibition of state III respiration by carbenoxolone raises the question about the cellular ATP levels. In the present work the tissue contents of AMP, ADP and ATP were measured before and after 20 minutes of 300 μM carbenoxolone

infusion. The results are shown in Table 2 and they reveal significant changes in the ATP and AMP levels. The levels of ATP were reduced while AMP levels were increased. These changes also resulted in a significant decrease in the ATP to AMP ratio and small decreases in the total adenine nucleotide contents. The ADP levels and the ATP to ADP ratio were not significantly affected by carbenoxolone.

The effects of 200 μ M and 300 μ M carbenoxolone on the adenine nucleotide contents were also evaluated in livers of 24-h fasted rats during 2 mM lactate infusion. The results are shown in Table 3. The ATP level was reduced by 49.9% in the presence of 200 μ M carbenoxolone. This reduction in the ATP level was only partly counterbalanced by an increase in the AMP level so that significant reductions in the total adenine nucleotide content took place. Both the ATP/AMP and the ATP/ADP ratios were reduced. At the concentration of 300 μ M carbenoxolone caused strong reductions in the ATP and AMP levels with a 68% reduction in the total adenine nucleotide content.

4. Discussion

The results of the liver perfusion experiments revealed that carbenoxolone affects the hepatic metabolism probably acting as an inhibitor of the mitochondrial energy transduction. The following observations support this conclusion: (a) carbenoxolone increased glycolysis and glycogenolysis, which are expected compensatory phenomena for the decreased mitochondrial ATP formation; (b) gluconeogenesis, a biosynthetic route strictly dependent on energy in the form of ATP was inhibited; (c) the cellular ATP levels were decreased by carbenoxolone; this action was more pronounced in livers from fasted rats in the presence of lactate as the gluconeogenic substrate; (d) the AMP levels were increased while the total adenine nucleotide content as well as the ATP/AMP ratio were decreased in both fed and fasted conditions. It must be mentioned that the effects of carbenoxolone on carbohydrate metabolism are comparable to those caused by classical inhibitors of oxidative phosphorylation, such as cyanide, 2,4-dinitrophenol and atractyloside [26-29]. Concerning oxygen uptake its effect is more similar to that of 2,4-dinitrophenol [30]. The latter is an uncoupling agent, which increases oxygen uptake in a wasteful manner, inhibiting ATP synthesis and increasing ATP hydrolysis within the mitochondria [31].

The data obtained with isolated mitochondria corroborate the conclusion that carbenoxolone impairs energy metabolism probably acting as an uncoupler of oxidative phosphorylation. The uncoupling action is indicated by its effects on mitochondrial respiration and ATPase activity, namely stimulation of state IV respiration and substrate respiration, decrease of the respiratory control ratio and increase of ATP hydrolysis in intact and coupled mitochondria [11,24]. The effects of car-benoxolone on the ATPase activity of different mitochondrial preparations, however, suggests that, in addition to the uncoupling action, the drug could also be a weak inhibitor of the ATP/ADP exchange system [32]. In this respect it should be noted that in intact coupled mitochondria stimulation of ATPase was the predominant action of carbenoxolone, but a small inhibition of this activity occurred in uncoupled mitochondria at the highest carbenoxolone concentrations. In freeze-thawing disrupted mitochondria, on the other hand, no action on the ATPase activity was found. In the latter preparation ATP has free access to the ATPase, whereas in fully uncoupled mitochondria transport is a

necessary and rate-limiting event. In coupled mitochondria, on the other hand, ATP hydrolysis is low and a weak inhibition of ATP/ADP exchange will not avoid a net stimulation of the ATPase activity.

It should be noted that the chemical structure of carbenoxolone bears the most important general characteristic that can be expected for protonophorous uncouplers [31]: a lipophilic structure formed by several C₆-rings combined with two carboxylic groups [7]. Such compounds usually interact and bind very effectively to cellular membranes. Indications that carbenoxolone binds to the hepatic cell membranes can be found in the kinetics of both the development and reversion of its effects. The various inhibitions and stimulations of carbenoxolone take more time to develop than those of cyanide, for example, which is a non-lipophilic inhibitor of energy transduction [30,33]. Furthermore, the reversion of the effects on glycogen catabolism after cessation of the carbenoxolone infusion is clearly preceded by a lag phase suggesting that it takes a certain time for the compound to be removed from the cellular space. This phenomenon is similar to that one found for propofol, a lipophilic anaesthetic whose binding to the intracellular membranes has been investigated in detail [24]. The increase in lactate dehydrogenase release into the effluent promoted by carbenoxolone perfusate is an additional indication that the compound is able to interact with biological membranes.

The toxic action of carbenoxolone at the mitochondrial level was previously suggested by a recent study by Salvi et al. [7], which have emphasized its ability to collapse the mitochondrial membrane potential and to induce hydrogen peroxide generation and mitochondrial permeability transition by means of oxidative stress. According to this study, carbenoxolone could also trigger the apoptotic pathway, since the above events would result in the loss of cytochrome c, an action which is strictly dependent on Ca²⁺. The present study complements these observations in isolated mitochondria and extends the observational field for some of the consequences for the whole cell. In the present study a wide range of carbenoxolone concentrations was investigated (20 to 300 μM), whereas the experiments of Salvi et al. [7] were done with a single concentration of 10 μM. It should be noted, however, that several of the effects observed in the present study were already significant at fairly low carbenoxolone concentrations. For example, the ATPase activity of intact mitochondria was 100% increased at 21.8 μM. If one takes this phenomenon as an indicator for the uncoupling activity

it becomes clear that the collapse of the mitochondrial membrane potential observed by Salvi et al. [7] is, partly at least, due to the uncoupling action of carbenoxolone. Furthermore, in the perfused liver, inhibition of gluconeogenesis from lactate was already significant with carbenoxolone 25 μ M, indicating that in the intact cell carbenoxolone is equally able to affect basic and important metabolic pathways at relatively low concentrations. The results of the present study could explain, partly at least, the earlier observations of Laubenthal [9] which reported evidence of liver damage by carbenoxolone during prolonged treatment. Abnormal bromosulphophthalein clearance and increased levels of hepatic enzymes, including lactate dehydrogenase, were found. The excretion of conjugated bromosulphophthalein into bile is an energy-dependent process and it is likely to be negatively affected when the energy metabolism is impaired [34]. Furthermore, the release of enzymes from the cell generally occurs when energy metabolism is impaired, especially when this action is exerted by agents possessing lipophilic structures [17,35]. Thus, carbenoxolone, by combining its activity on energy metabolism with its lipophilic nature can be expected to be especially effective in producing structural changes in biological membranes even though these changes are fully reversible [9].

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Table 1

The action of carbenoxolone on mitochondrial respiration driven by succinate and β -hydroxybutyrate in the presence and absence of exogenously added ADP. Mitochondria were isolated and assayed as described in Material and methods. Incubations were done in the presence of substrate (10 mM) as indicated. Data are the means \pm standard errors. Statistical significance relative to the controls is indicated by asterisks (variance analysis with post-hoc Newman-Keuls testing; $p < 0.05$)

Carbenoxolone (μM)	β-Hydroxybutyrate (n=8)		Succinate (n=7)	
	ADP/O	Respiratory control ratio	ADP/O	Respiratory control ratio
0	2.85 \pm 0.27	6.58 \pm 0.55	1.66 \pm 0.13	5.97 \pm 0.62
20	2.80 \pm 0.20	4.07 \pm 0.55*	1.90 \pm 0.16	5.55 \pm 0.53
50	2.55 \pm 0.28	3.42 \pm 0.35*	1.81 \pm 0.21	4.65 \pm 0.49
100	2.49 \pm 0.39	2.94 \pm 0.35*	1.76 \pm 0.22	3.49 \pm 0.37*
160	1.57 \pm 0.54*	1.21 \pm 0.11*	1.66 \pm 0.25	2.39 \pm 0.58*
200	—	1.02 \pm 0.02*	1.51 \pm 0.31	1.53 \pm 0.44*

Table 2***Influence of carbenoxolone on hepatic contents of adenine nucleotides in substrate-free perfused livers of fed rats.***

Livers were perfused in an open system as described in Materials and methods. Carbenoxolone (300 μM) was infused and at 30 minutes after starting infusion the livers were freeze-clamped in liquid nitrogen and extracted with perchloric acid. The neutralized extracts were used for determination of the adenine nucleotides by means of standard enzymatic procedures. Control determinations were done with livers that were freeze-clamped at the same perfusion time but without carbenoxolone infusion. The data are means \pm mean standard errors. Values labeled with an asterisk are statistically different from the corresponding control values according to Student's t test ($p < 0.05$).

Conditions	ATP	ADP	AMP	AMP+ADP+ATP	ATP/ADP ratio	ATP/AMP ratio
	$\mu\text{mol} \times (\text{g liver wet weight})^{-1}$					
Control (n = 4)	1.92 ± 0.06	0.57 ± 0.05	0.22 ± 0.03	2.72 ± 0.07	3.49 ± 0.42	8.92 ± 0.98
Carbenoxolone (300 μM) (n = 4)	1.25 $\pm 0.18^*$	0.60 ± 0.07	0.51 $\pm 0.05^*$	2.36 $\pm 0.10^*$	2.27 ± 0.53	2.59 $\pm 0.54^*$

Table 3

Influence of carbenoxolone on hepatic contents of adenine nucleotides in the presence of lactate 2.0 mM. Livers from fasted rats were perfused in an open system as described in Materials and methods. Lactate 2.0 mM was infused at 10 minutes and carbenoxolone (200 μ M or 300 μ M) were infused 20 minutes after the beginning of lactate infusion during 30 minutes. The livers were freeze-clamped in liquid nitrogen and the adenine nucleotides were extracted with cold perchloric acid. Determination was accomplished by standard enzymatic procedures. Control determinations were done with livers that were freeze-clamped at the same perfusion time in the presence of lactate but without carbenoxolone infusion. The data are means \pm mean standard errors. Statistical significance relative to the controls is indicated by asterisks (variance analysis with post-hoc Newman-Keuls testing; $p < 0.05$)

Conditions	ATP	ADP	AMP	AMP +	ADP + ATP	ATP/ADP ratio	ATP/AMP ratio
	$\mu\text{mol} \times (\text{g liver wet weight})^{-1}$						
Control (n = 3)	1.63 ± 0.16	0.38 ± 0.03	0.25 ± 0.04		2.26 ± 0.22	4.28 ± 0.27	7.23 ± 1.67
Carbenoxolone (200 μM) (n = 6)	0.83 $\pm 0.07^*$	0.51 ± 0.05	0.41 $\pm 0.04^*$		1.76 $\pm 0.10^*$	1.73 $\pm 0.27^*$	2.26 $\pm 0.39^*$
Carbenoxolone (300 μM) (n = 3)	0.39 $\pm 0.05^*$	0.22 ± 0.06	0.12 ± 0.02		0.72 $\pm 0.06^*$	2.04 $\pm 0.53^*$	3.58 $\pm 0.81^*$

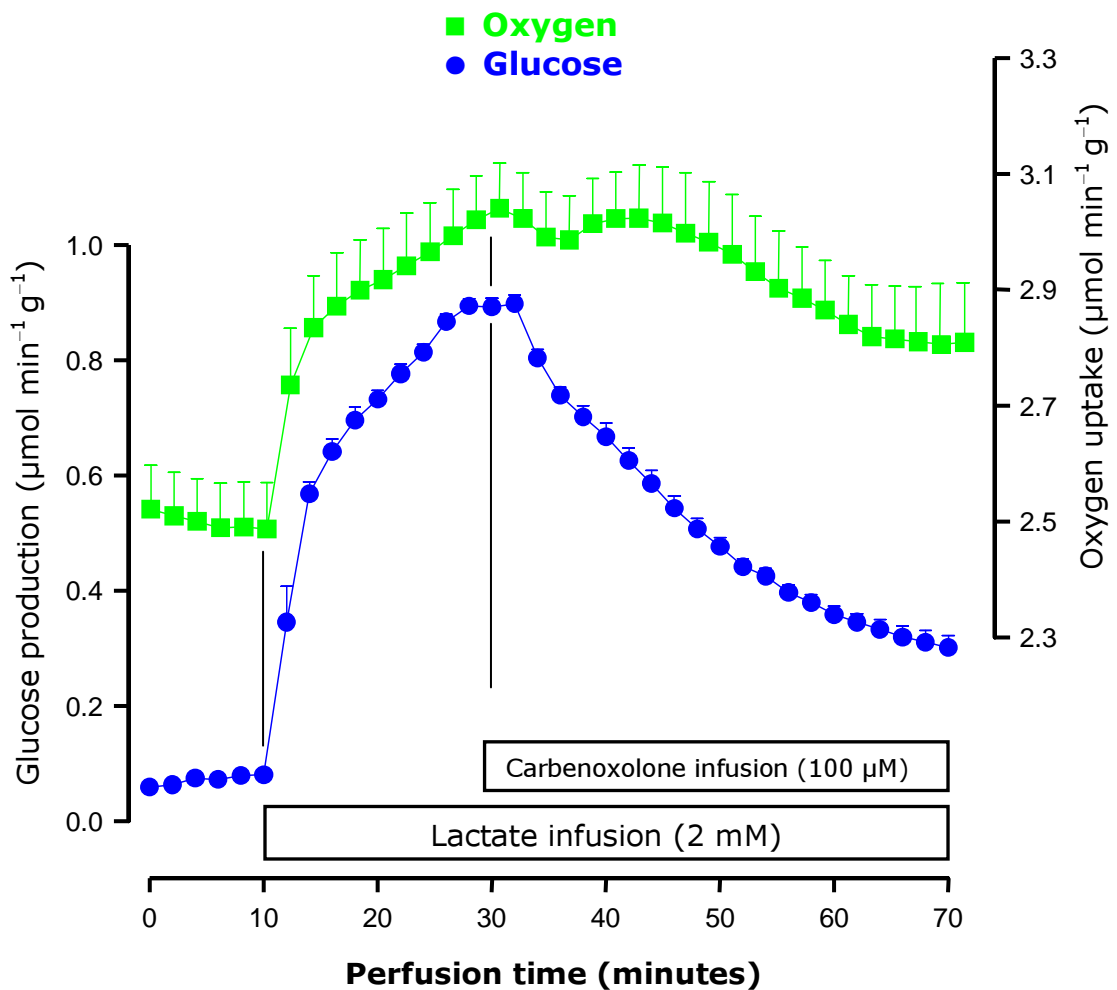


Figure 1. **Time course of the effects of 100 μM carbenoxolone on gluconeogenesis from lactate and oxygen uptake in livers from fasted rats.** Samples of the effluent perfusate were withdrawn for the measurement of glucose. Oxygen in the venous perfusate was monitored polarographically. The lactate and carbenoxolone infusion times are indicated by horizontal bars. The data represent the means ($\pm\text{S.E.M.}$) of 4 liver perfusion experiments.

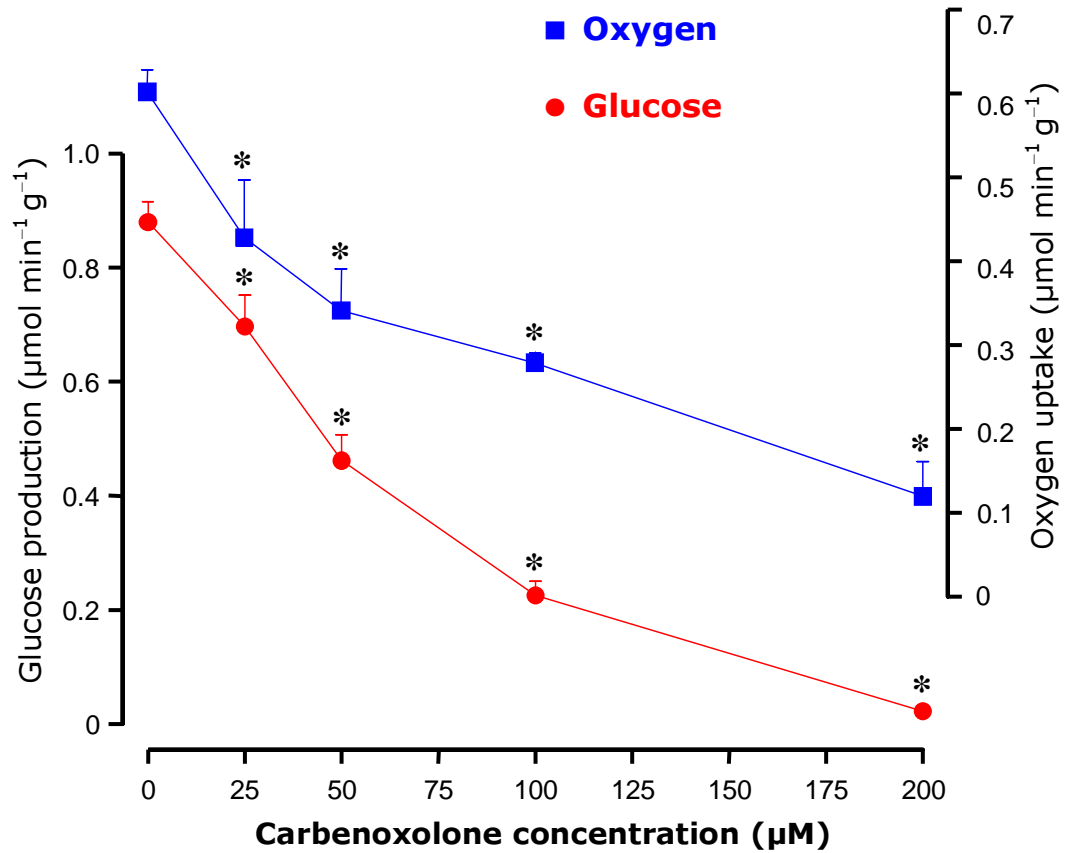


Figure 2. **Concentration dependence of the action of carbenoxolone on glucose production from lactate and Δ oxygen consumption in livers from fasted rats.** The data were obtained from experiments of the kind illustrated by Figure 1. The control values (zero carbenoxolone) correspond to the rates found in the presence of lactate just before the onset of carbenoxolone infusion (30 minutes perfusion time) minus the basal rates (i.e., before the onset of lactate infusion). Rates in the presence of lactate + carbenoxolone were evaluated at 70 minutes perfusion time and also subtracted from the same basal rates. Each datum point represents the mean (\pm S.E.M.) of 3–5 liver perfusion experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Newman-Keuls testing ($p < 0.05$).

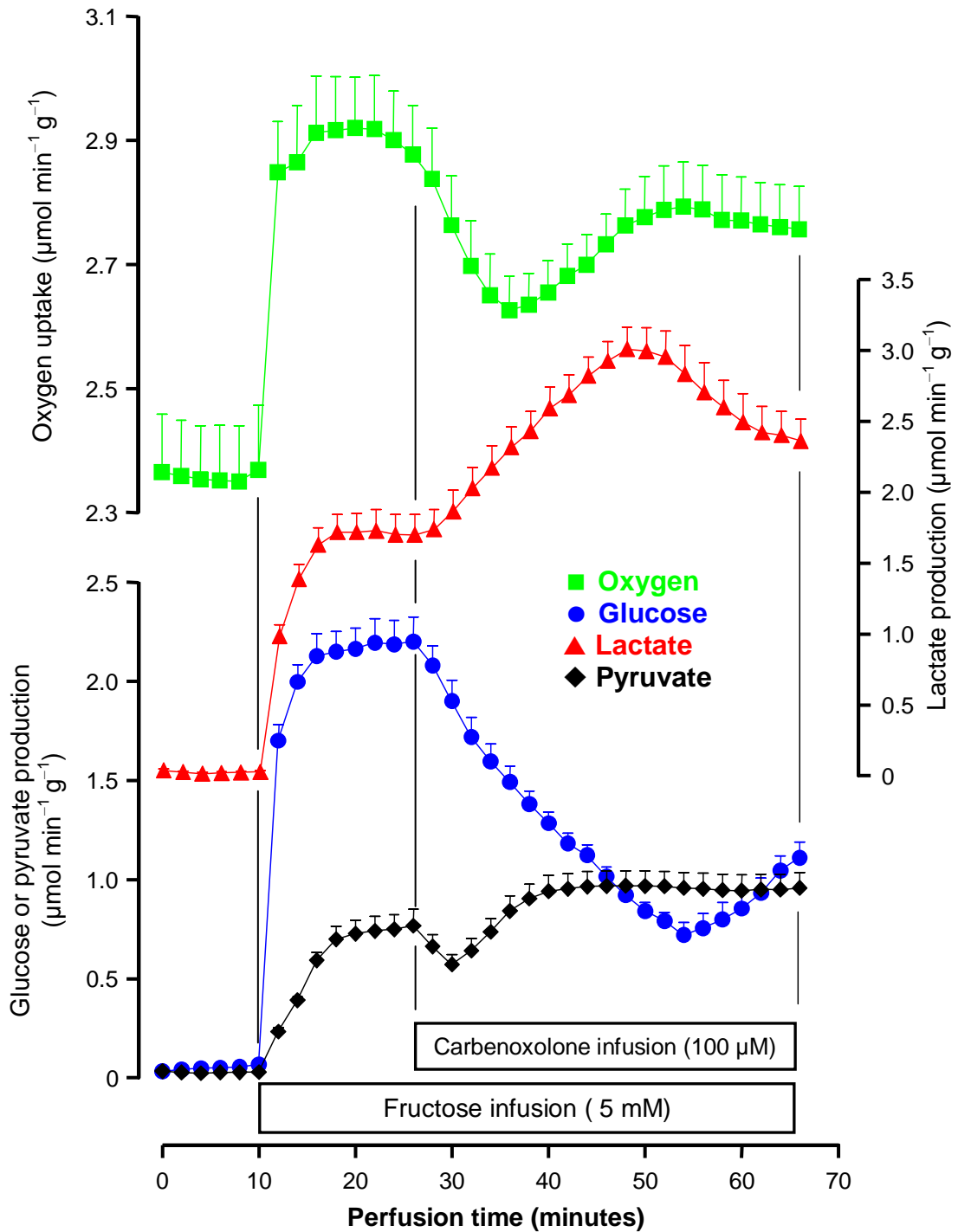


Figure 3. **Time course of the effects of 100 μM carbenoxolone on gluconeogenesis from fructose and oxygen uptake in livers from fasted rats.** Samples were collected in 2 minutes intervals and aliquots were used for the enzymatic measurement of glucose, L-lactate and pyruvate. Oxygen in the venous perfusate was monitored polarographically. The fructose and carbenoxolone infusion times are indicated by horizontal bars. The data represent the means (\pm S.E.M.) of 4 liver perfusion experiments.

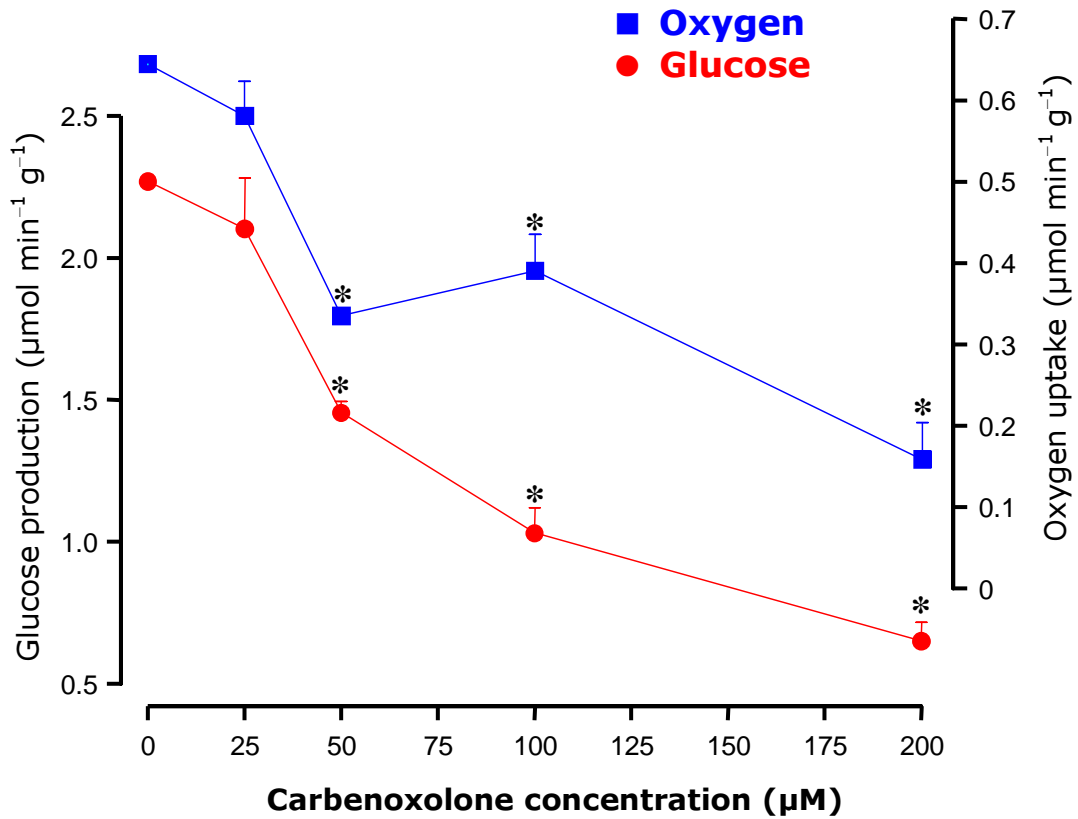


Figure 4. **Concentration dependence of the action of carbenoxolone on the metabolic fluxes resulting from fructose metabolism in livers from fasted rats.** The data were obtained from experiments of the kind illustrated by Figure 3 with 5 mM fructose as the gluconeogenic substrate. The control values (zero carbenoxolone) correspond to the rates found in the presence of fructose just before the onset of carbenoxolone infusion (30 minutes perfusion time) minus the basal rates (i.e., before the onset of fructose infusion). Rates in the presence of fructose + carbenoxolone were evaluated at 66 minutes perfusion time and also subtracted from the same basal rates. Each datum point represents the mean (\pm S.E.M.) of 4 liver perfusion experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Newman-Keuls testing ($p < 0.05$).

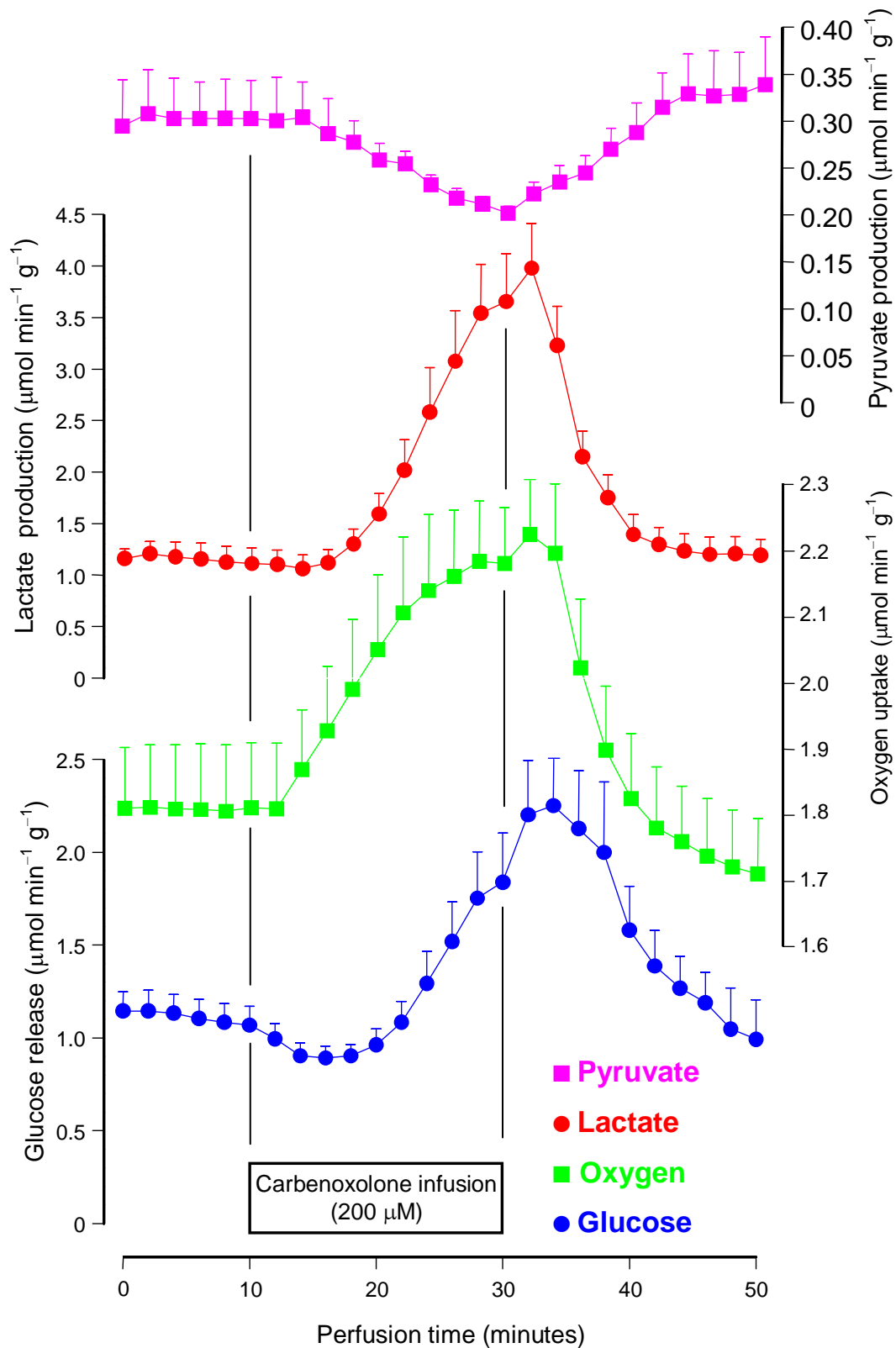


Figure 5. **Time course of the effects of 200 μM carbenoxolone on glycogen catabolism and oxygen uptake in livers from fed rats.** Samples of the effluent perfusate were withdrawn for metabolite assay. Oxygen in the venous perfusate was monitored polarographically. The carbenoxolone infusion time is indicated by the horizontal bar. Data represent the mean (\pm S.E.M.) of 4 liver perfusion experiments.

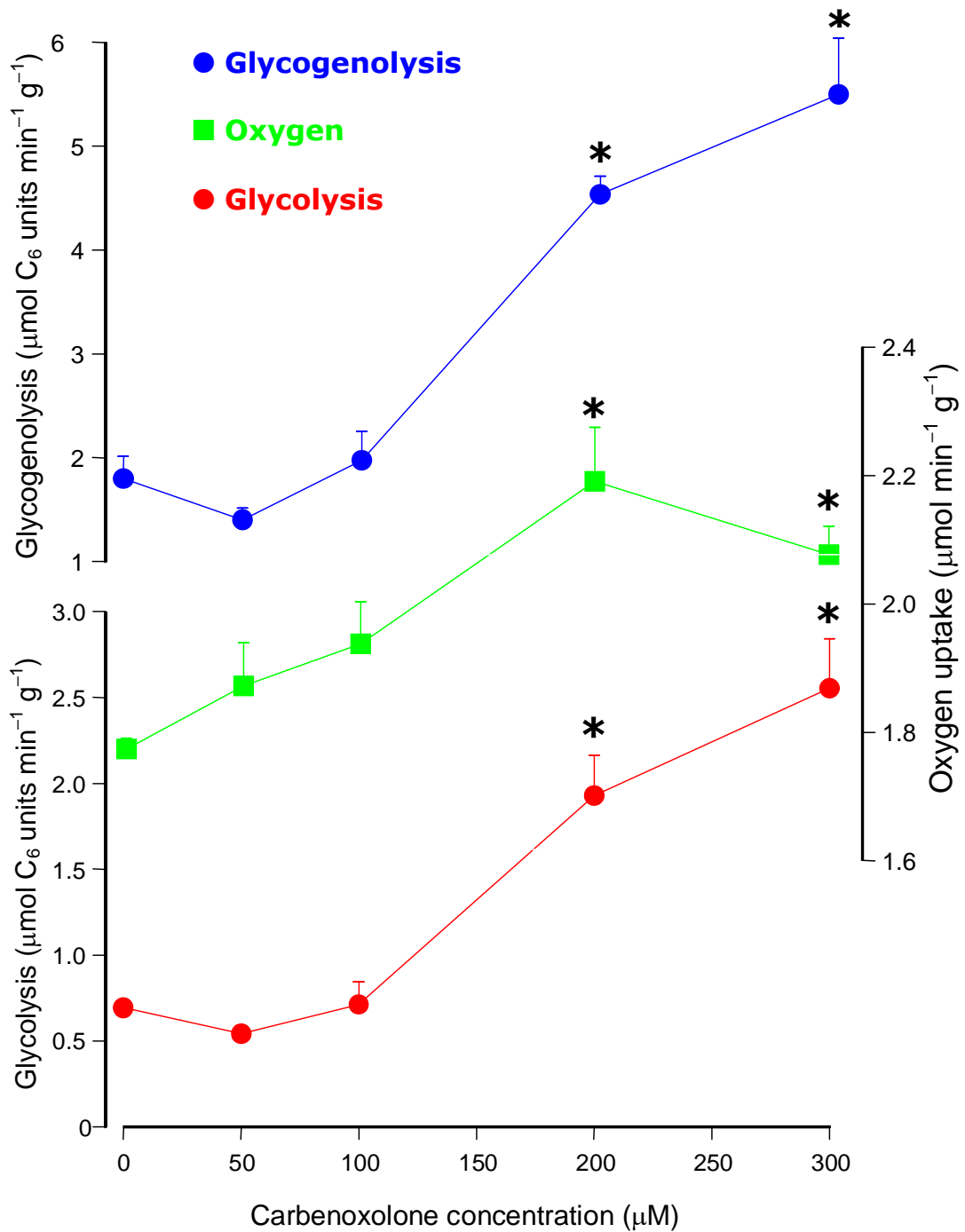


Figure 6. **Concentration dependence of the action of carbenoxolone on glycolysis, glycogenolysis and oxygen consumption in livers from fed rats.** The data were obtained from experiments of the kind illustrated by Figure 5. The control values (zero carbenoxolone) correspond to the basal rates found before the onset of carbenoxolone infusion (10 minutes perfusion time). Rates in the presence of carbenoxolone were evaluated at 30 minutes perfusion. Glycogenolysis and glycolysis were calculated from glucose, lactate and pyruvate production and expressed as glucosyl units: glycogenolysis = glucose + $\frac{1}{2}$ (lactate + pyruvate); glycolysis = $\frac{1}{2}$ (lactate plus pyruvate). Each datum point represents the mean (\pm S.E.M.) of 4 liver perfusion experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with *post hoc* Newman-Keuls testing ($p < 0.05$).

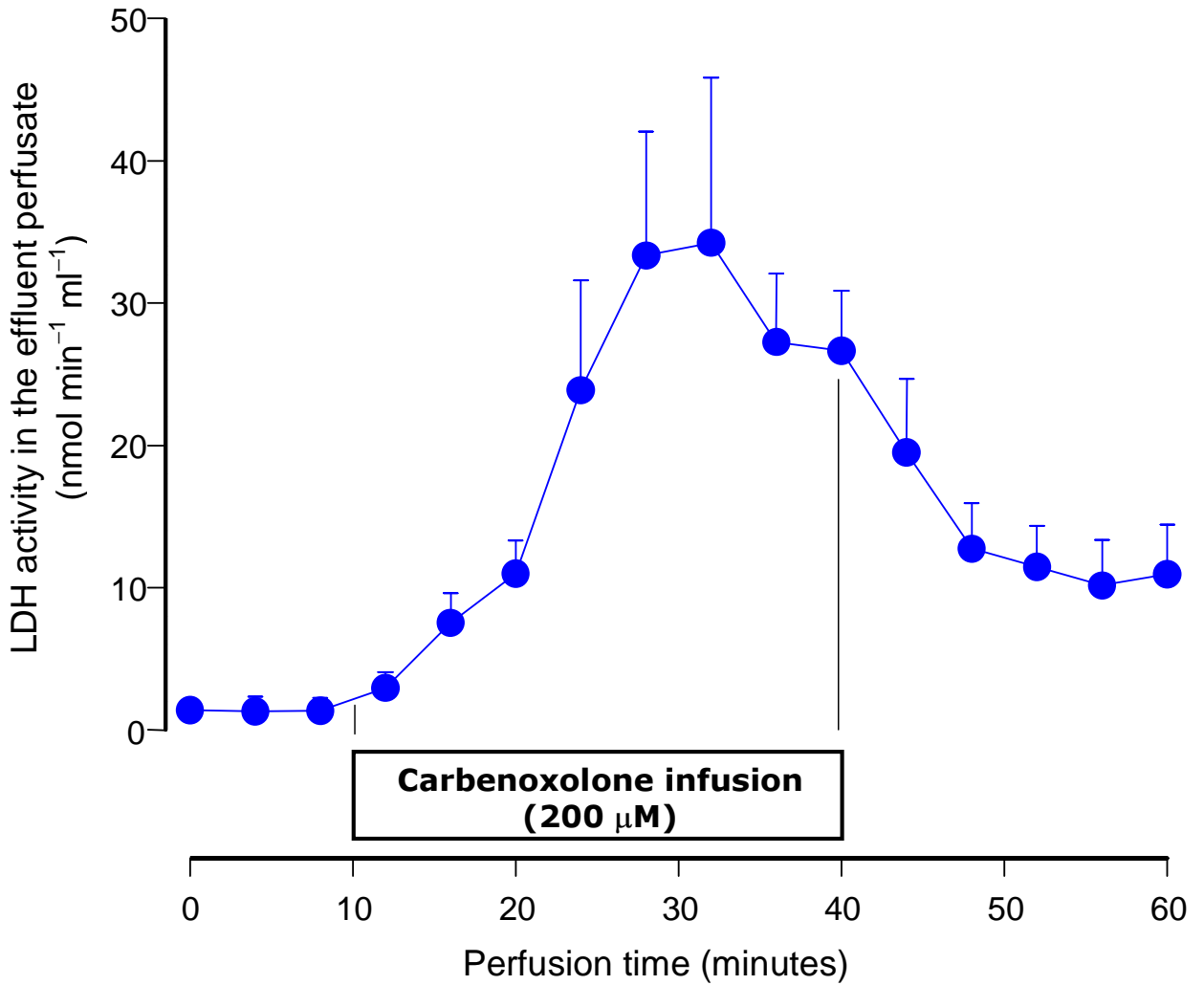


Figure 7. **Effects of carbenoxolone on lactate dehydrogenase release into the outflowing perfusate.** Livers from fed rats were perfused as described in Materials and Methods. Samples of the effluent perfusate were collected in 4 minute intervals and used for lactate dehydrogenase (LDH) assay. Data are the means \pm mean standard errors of 4 liver perfusion experiments.

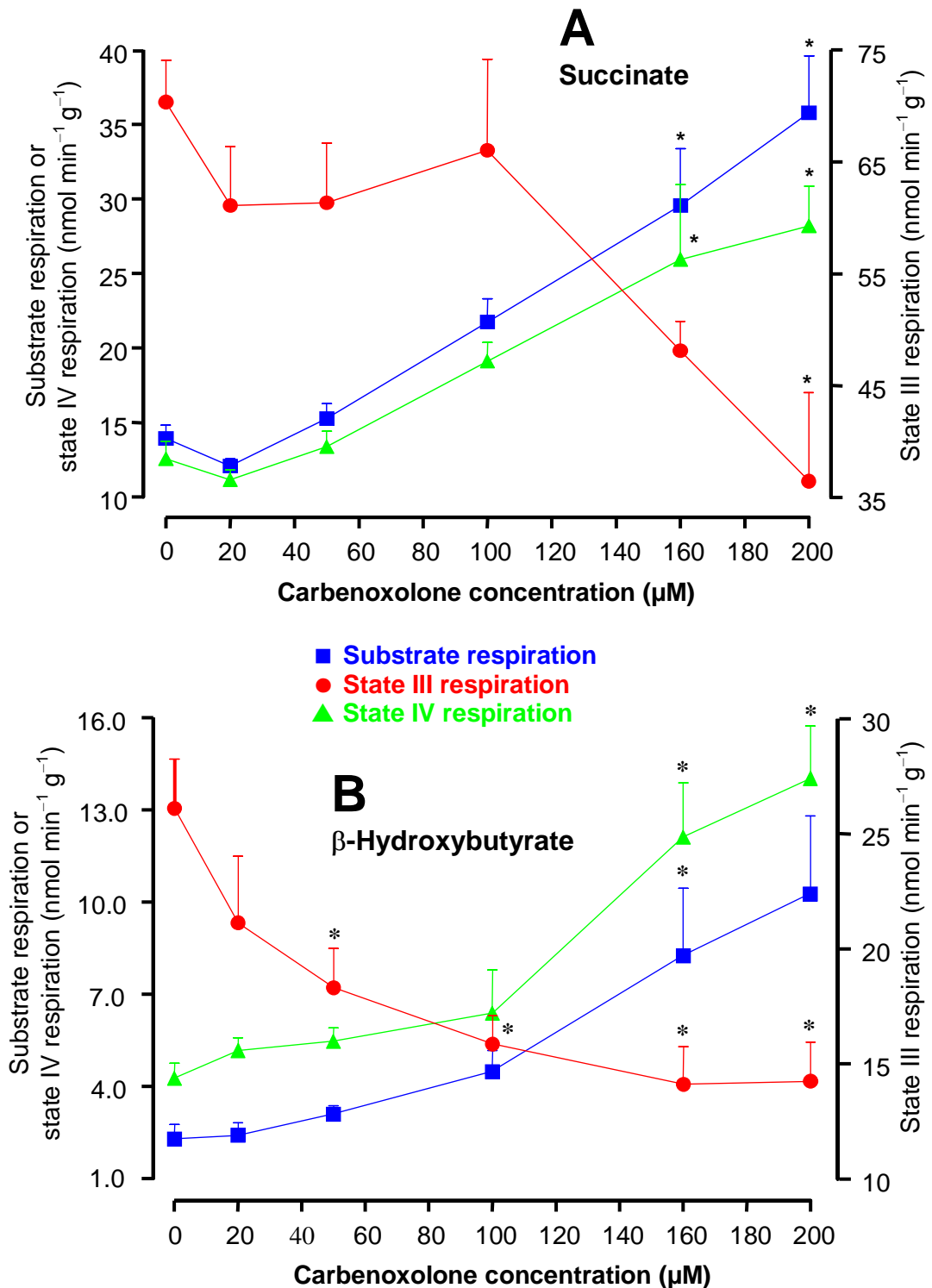


Figure 8. Effects of carbenoxolone on the respiratory activity of isolated rat liver mitochondria. Mitochondria (0.25-2.5 mg/ml) were added to the reaction medium in the closed vessel of the oxygraph. The reaction was initiated by the addition of succinate (A) or β -hydroxybutyrate (B) and the oxygen consumption was followed polarographically for 5 min. After this time 0.25-0.5 nmol of ADP were added. Rates of oxygen consumption were computed from the slopes of the polarographic records. Each datum point is the mean \pm SEM of four independent experiments. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with *post hoc* Newman-Keuls testing ($p < 0.05$).

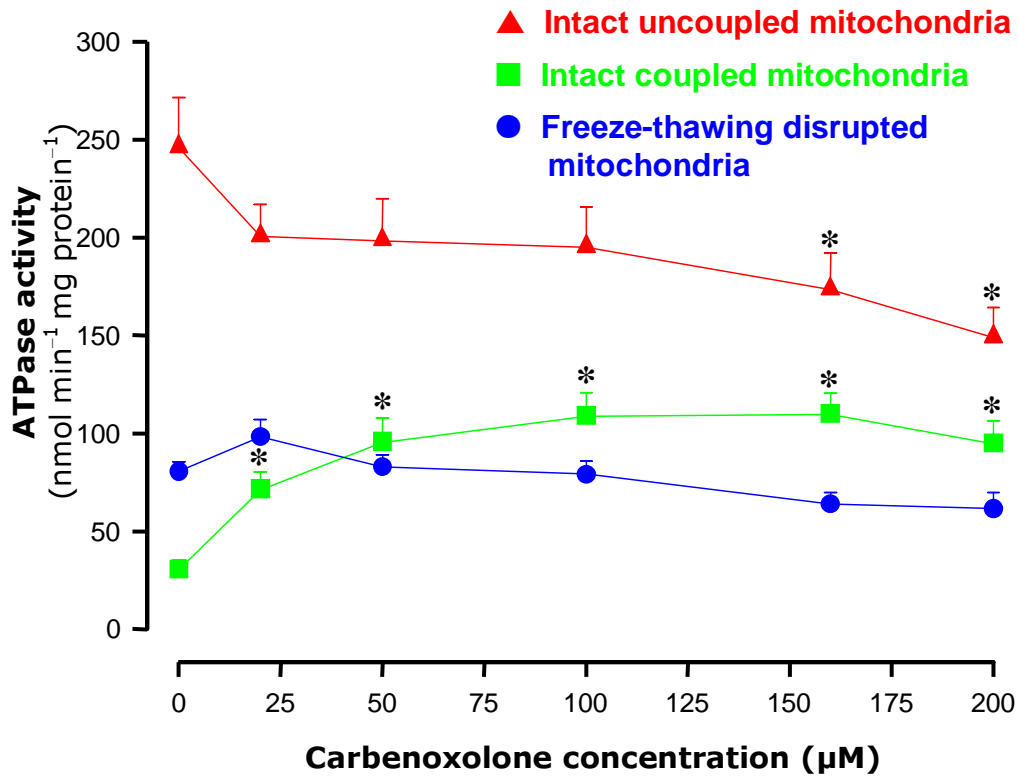


Figure 9. **Effects of carbenoxolone on the ATPase activity of coupled, uncoupled and disrupted mitochondria.** The mitochondria were incubated at 37°C in reaction medium as described in Materials and methods. Each assay point represents the mean of eight (coupled mitochondria), seven (0.2 mM 2,4-dinitrophenol uncoupled mitochondria) and eight (freeze-thawing disrupted mitochondria) independent experiments and each datum point is the mean \pm SEM. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with *post hoc* Newman-Keuls testing ($p < 0.05$).