Biochem. J. (1991) 280, 663–669 (Printed in Great Britain)

Inhibition of gluconeogenesis in isolated rat hepatocytes after chronic treatment with phenobarbital

Doriane ARGAUD,*‡ Serge HALIMI,† Françoise CATELLONI* and Xavier M. LEVERVE*
*Laboratoire de Thérapeutique, Université Joseph Fourier, Bât. 72 Biologie, BP 53X, 38041 Grenoble-Cedex, and
†Service de Diabétologie, Hôpital A. Michallon, BP 217, 38043 Grenoble-Cedex 9, France

Gluconeogenesis was studied in hepatocytes isolated from phenobarbital-pretreated rats fasted for 24 h. In closed vial incubations, glucose production from lactate (20 mmol/l) and pyruvate (2 mmol/l), alanine (20 mmol/l) or glutamine (20 mmol/l) was suppressed by about 30–45%, although glycerol metabolism was not affected. In hepatocytes perifused with lactate and pyruvate (ratio 10:1), glucose production was inhibited by 50%, even at low gluconeogenic flux. From the determination of gluconeogenic intermediates at several steady states of gluconeogenic flux, we have found a single relationship between phosphoenolpyruvate and the rate of glucose production ($J_{glucose}$), and two different curves between cytosolic oxaloacetate and $J_{glucose}$ in controls and in phenobarbital-pretreated hepatocytes. By using 3-mercaptopicolinate to determine the flux control coefficient of phosphoenolpyruvate carboxykinase we found that phenobarbital pretreatment led to an increase in this coefficient from 0.3 (controls) to 0.8 (phenobarbital group). These observations were confirmed by the finding that the activity of phosphoenolpyruvate carboxykinase was decreased by 50% after phenobarbital treatment. Hence we conclude that the inhibitory effect of phenobarbital on gluconeogenesis is due, at least partly, to a decrease in the flux through phosphoenolpyruvate carboxykinase.

INTRODUCTION

Chronic phenobarbital treatment is associated with an increase in smooth endoplasmic reticulum (SER) membranes and with modifications of some liver microsomal enzymes located in the SER; in particular, an enhancement of NADPH-utilizing mixed function oxidation, the major pathway of detoxification of drugs and many endogenous substances, is observed (Conney, 1967; Remmer, 1972; Schulte-Hermann, 1974).

A decrease in plasma glucose and an improvement in sensitivity to insulin have been shown in diabetic patients after chronic phenobarbital treatment (Lathela *et al.*, 1984, 1985, 1986). Such a fall in plasma glucose after chronic phenobarbital treatment may be due to a decrease in the glucogenic rate, since excessive hepatic glucose production is one of the major features in diabetic patients (Best *et al.*, 1982). Karvonen *et al.* (1989) have shown in lean female Zucker rats that phenobarbital stimulates the activities of NADPH-dependent enzymes, such as glucose-6-phosphate dehydrogenase and malic enzyme. Orrenius and co-workers (Orrenius *et al.*, 1965; Orrenius & Ericsson, 1966) have reported that chronic phenobarbital treatment in rats decreases the activity of the glucose-6-phosphatase (EC 3.1.3.9) bound to SER. Scholz *et al.* (1973), using the non-recirculating perfused liver model, have observed inhibition of gluconeogenesis from lactate following stimulation by aminopyrine of the NADPH-utilizing mixed function oxidation system that is induced by phenobarbital pretreatment in rats.

The purpose of the present work was to investigate the effects of phenobarbital pretreatment on gluconeogenesis in isolated hepatocytes. Moreover, by measuring the intracellular gluconeogenic intermediates it is possible to locate the effect of such chronic treatment, since many steps have been reported to be affected: glucose-6-phosphatase (Orrenius & Ericsson, 1966; Sotaniemi *et al.*, 1984; Karvonen *et al.*, 1987), pyruvate carboxylase (EC 6.4.1.1) (Scholz *et al.*, 1973) and NADPH-dependent enzymes (Karvonen *et al.*, 1989). We found a dramatic decrease in glucose synthesis from physiological substrates, e.g. lactate/pyruvate, alanine and glutamine, although glycerol metabolism was not affected. The inhibitory effect on gluconeogenesis was obtained without addition of exogenous substrates (e.g. aminopyrine) for NADPH-utilizing mixed function oxidation. Using perifused hepatocytes, we report here that the main gluconeogenic step influenced by such pretreatment may be phosphoenolpyruvate carboxykinase (EC 4.1.1.32).

MATERIALS AND METHODS

Male Wistar rats fed with stock pellets given *ad libitum* were used for this study. Chronic phenobarbital induction was obtained with an oral dose of 100 mg/kg per day for 15 days as reported previously by Marshall & McLean (1969). Drinking water was fully available to all animals, but for those receiving oral sodium phenobarbital, an aqueous solution of the drug in tap water was the only source of water. The water intake, given either as water or as a 1 mg/ml solution of sodium phenobarbital, was approx. 10 ml/100 g per day (giving a dose of 100 mg/kg per day). During the 15 days of treatment, the weight gained by the phenobarbital-treated rats was the same as in the control group. As reported previously by Marshall & McLean (1969), pretreated rats were only lethargic for the first 2 days. On the day of experiment, both groups had the same weight: controls, 245 ± 13 g; phenobarbital-treated, 244 ± 12 g.

After 15 days of sodium phenobarbital pretreatment, rats (200–250 g) were fasted for 24 h. They were then anaesthetized with sodium pentobarbital intraperitoneally (112.5 mg/kg for control rats; 155 mg/kg for phenobarbital-pretreated rats). Hepatocytes were isolated according to the method of Berry & Friend (1969), as modified by Groen *et al.* (1982*a*).

Chronic phenobarbital induction of microsomal protein was shown by determination of levels of cytochrome *P*-450, a mixed function oxidase. Cytochrome *P*-450 was measured by the method of Omura & Sato (1964) using a Kontron/UVIKON 810

Abbreviations used: SER, smooth endoplasmic reticulum; 3-MPA, 3-mercaptopicolinic acid.
‡ To whom correspondence should be addressed.

Table 1. Effect of phenobarbital treatment on gluconeogenesis in hepatocytes incubated in closed vials

Hepatocytes (10 mg dry mass/ml) isolated from 24 h-starved male Wistar rats pretreated or not with sodium phenobarbital (PB) (100 mg/kg per day) for 15 days were incubated in 2.8 ml of Krebs bicarbonate buffer (pH 7.4) containing BSA oleate (2 mmol/l). As indicated, the substrate additions were lactate (20 mmol/l) + pyruvate (2 mmol/l), alanine (20 mmol/l) or glutamine (20 mmol/l). Incubations with glycerol (20 mmol/l) were performed without BSA oleate. The amounts of glucose production were determined every 15 min during a 60 min incubation. Results are expressed in μmol/g dry mass and are means ± S.E.M. Values in parentheses are percentage inhibition ($n = 15$) caused by phenobarbital treatment. Statistical comparisons were made using Student's t test for unpaired samples; $^{**}P < 0.01$, $^{***}P < 0.001$.

	Glucose production (μmol/g)							
	Lactate/pyruvate		Alanine		Glycerol		Glutamine	
Time (min)	Control	PB	Control	PB	Control	PB	Control	PB
15	90±6	69±4 (23%)**	21±2	11±0.2 (47%)***	49±3	47±1	17±1	10±0.3 (46%)***
30	217±10	146±7 (33%)***	64±3	38±1 (40%)***	95±5	97±3	51±2	27±2 (47%)***
45	328±14	215±9 (34%)***	119±4	72±2 (39%)***	143±8	137±4	88±4	47±4 (46%)***
60	431±17	308±13 (29%)***	180±7	110±3 (39%)***	200±10	189±5	128±5	73±5 (43%)***

spectrophotometer. For this determination, isolated hepatocytes were diluted with phosphate buffer (final concentration 0.05 mol/l, pH 7.4) so that 1 ml of suspension contained 1.7 mg of dry mass/ml or 1 mg of protein/ml (1.8 ml of buffer + 50 μl samples). Cytochrome P-450 increased dramatically in the phenobarbital-pretreated group (450 ± 50 versus 150 ± 10 nmol/g dry mass, $P < 0.001$). These values are in good agreement with the data of Marshall & McLean (1969), showing that all pretreated rats were induced.

Hepatocytes (final concentration 10 mg dry mass/ml) were incubated for 1 h in 2.8 ml of Krebs bicarbonate buffer (NaCl 120 mmol/l, KCl 4.8 mmol/l, KH_2PO_4 1.2 mmol/l, $MgSO_4$ 1.2 mmol/l, $NaHCO_3$ 24 mmol/l, Ca^{2+} 2.4 mmol/l, pH 7.4) containing various substrates, as indicated in the Figure legends; the gas atmosphere was O_2/CO_2 (19:1) and the temperature was 37 °C. Samples (0.3 ml) of the cell suspension were taken every 15 min and quenched with $HClO_4$ (final concentration 5%, w/v). After centrifugation, the supernatant was neutralized with 2 M-KOH/0.3 M-Mops for subsequent determination of metabolites.

Liver cells were perifused according to the method of van der Meer & Tager (1976) with the modification described in Groen *et al.* (1982*a*). The perifusion fluid was Krebs bicarbonate buffer containing Ca^{2+} (1.3 mmol/l), 0.1% BSA and oleate (0.1 mmol/l). For each steady state, intracellular metabolites were determined as follows. A 0.7 ml sample taken from the perifusion chamber was placed on top of a layer of silicone oil (Rhodorsil 640 V 100) and centrifuged (13500 ***g***, 15 s) through the layer of oil into $HClO_4$ (final concentration 10%, w/v). Metabolites were measured in neutralized protein-free extracts. The concentration of cytosolic malate was measured after fractionation of the cells using the digitonin technique as described by Zuurendonk & Tager (1974) and modified by Groen *et al.* (1982*a*). The concentration of cytosolic oxaloacetate was subsequently calculated from the cytosolic malate concentration and the lactate/pyruvate ratio, assuming equilibrium for lactate dehydrogenase (EC 1.1.1.27) (Williamson *et al.*, 1967).

Phosphoenolpyruvate carboxykinase activity was determined after 15 days of sodium phenobarbital pretreatment. Enzyme activity was measured as described by Chang & Lane (1966) in frozen liver samples from 24 h-fasted rats. Protein concentration was determined using the Bio-Rad protein assay (Bio-Lab., München, Germany), with BSA as standard.

All metabolites were measured either spectrophotometrically or fluorimetrically using standard enzymic methods (Bergmeyer, 1970). Amino acids, glycerol, lactate and glucagon were purchased from Sigma Chemical Co. (Lyon, France). Pyruvate, collagenase A and enzymes were purchased from Boehringer (Meylan, France). 3-Mercaptopicolinic acid (3-MPA) was a gift from Dr. A. J. Meijer, University of Amsterdam. Oleate, dihydroxyacetone and digitonin were obtained from Merck (Lyon, France). Rhodorsil silicone oil was purchased from Rhône-Poulenc (Lyon, France).

Results are expressed as means ± S.E.M. Comparisons were made using Student's t test for unpaired samples.

RESULTS

Effect of phenobarbital treatment on gluconeogenesis from various substrates

When hepatocytes were incubated in closed vials without any exogenous substrate, glucose production from endogenous substrate was inhibited (19.25 ± 1.20 versus 12.42 ± 0.59 μmol/g dry mass after 1 h for the control and pretreated groups respectively, $P < 0.001$). Glucose production was linear over the last 45 min of incubation and the calculated fluxes were inhibited by phenobarbital: 0.34 ± 0.02 μmol/min per g dry mass for control rats versus 0.22 ± 0.01 μmol/min per g for pretreated rats ($P < 0.001$). This effect could be due either to decreased endogenous substrate production (e.g. a decrease in amino acid production from proteolysis), or to a decrease in their utilization in the gluconeogenic pathway.

The inhibitory effects of phenobarbital treatment on glucose production with various gluconeogenic substrates are summarized in Table 1. Phenobarbital treatment inhibited glucose production whether from lactate + pyruvate (10:1, mol/mol) (30%), alanine (40%) or glutamine (45%). This effect was highly significant and nearly constant with time after 15 min of incubation. No effect was observed when glycerol was the gluconeogenic substrate. From these data, we can conclude that the inhibition of gluconeogenesis following chronic phenobarbital treatment is a common feature for all the substrates we used, except for glycerol.

These results were obtained in closed vial incubations at saturating substrate concentrations. In order to investigate this phenomenon in a more physiological manner, we performed experiments using perifused hepatocytes. This method, described by van der Meer & Tager (1976), allows us to titrate the gluconeogenic flux by increasing the substrate concentrations from low to saturating concentrations. Moreover, at each steady state, by sampling cells from the chambers, gluconeogenic

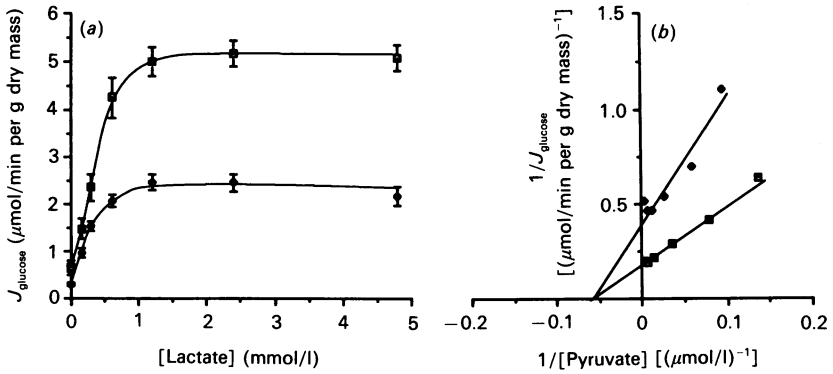

Fig. 1. Effect of phenobarbital treatment on lactate + pyruvate metabolism in perifused hepatocytes

Hepatocytes (approx. 240 mg dry mass in 12 ml) isolated from rats either treated (◆) or not (□) with sodium phenobarbital (100 mg/kg per day for 15 days), were perifused with BSA (0.1%) and oleate (0.1 mmol/l). The flow rate was 5 ml/min of Krebs bicarbonate buffer (pH 7.4), continuously gassed with O_2/CO_2 (19:1). Cells were titrated with lactate + pyruvate (10:1) as indicated in (*a*). The rate of glucose formation ($J_{glucose}$) was calculated from the glucose concentration measured in the perifusate. Results are expressed as means ± S.E.M. (control $n = 5$, treated $n = 8$). (*b*) Lineweaver–Burk plot for the rate of glucose formation ($1/J_{glucose}$) as a function of the reciprocal concentration of pyruvate in the perifusate.

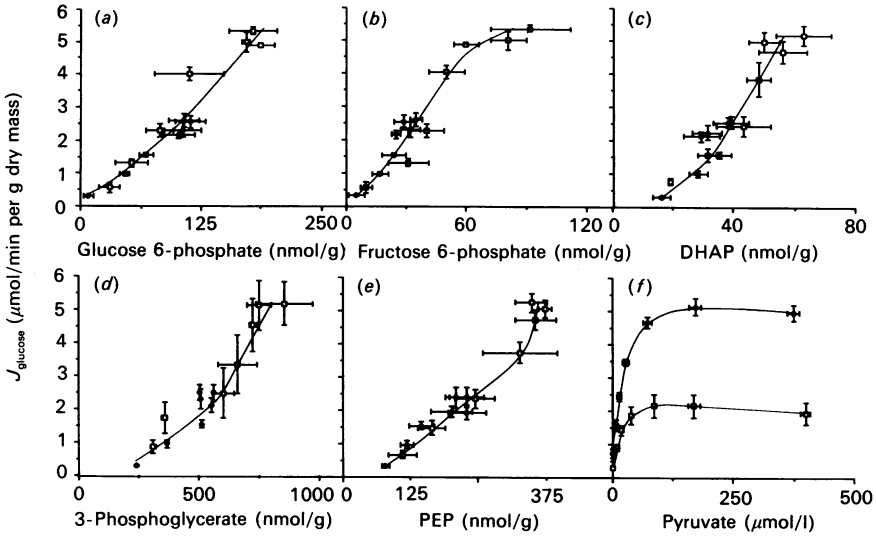

Fig. 2. Relationship between gluconeogenic flux and the concentrations of various intermediates

Cells from rats treated (◆) or not (□) with phenobarbital were perifused as described in Fig. 1. At each steady state, 0.7 ml samples of the cell suspension were removed from the perifusion vessel and centrifuged through a layer of silicone oil into $HClO_4$ (10%, w/v). Pyruvate (*f*) and glucose concentrations were measured in the perifusate. (*a*) Glucose 6-phosphate, (*b*) fructose 6-phosphate, (*c*) dihydroxyacetone phosphate (DHAP), (*d*) 3-phosphoglycerate and (*e*) phosphoenolpyruvate (PEP), were measured in the neutralized intracellular fraction and are given as nmol/g dry mass. The concentration of extracellular pyruvate is given in μmol/l. Results are given as means ± S.E.M. (*a* and *b*: control, $n = 3$; treated, $n = 6$; *c*: control, $n = 4$; treated, $n = 6$; *d*: control, $n = 2$; treated, $n = 5$; *e*: control, $n = 5$; treated, $n = 6$; *f*: control, $n = 5$; treated, $n = 6$.)

intermediates can be determined. By plotting intermediate concentrations and the gluconeogenic flux, some information concerning the step(s) involved in the inhibitory effect can be obtained. We chose lactate and pyruvate (in a ratio of 10:1) as substrates because of their great physiological importance in gluconeogenesis.

Effect of chronic phenobarbital treatment on gluconeogenesis from lactate + pyruvate in perifused hepatocytes

These experiments were carried out with oleate (0.1 mmol/l) in order to activate pyruvate carboxylase by providing acetyl-CoA, its allosteric activator (Groen *et al.*, 1983). In similar conditions these authors found a maximal value for the rate glucose production ($J_{glucose}$) of 4.7 μmol/min per g dry mass, which is very close to our results (Fig. 1). As shown in Fig. 1, a stronger inhibitory effect was observed when hepatocytes were perifused (50%) than in those incubated in closed vials (30%). This inhibitory effect was present even at low substrate concentrations (0.15 mmol/l). Moreover, the highest gluconeogenic rate was obtained at a lactate concentration of 1.2 mmol/l (Fig. 1) [$J_{glucose} = 4.99 \pm 0.3$ μmol/min per g dry mass ($n = 5$) for the control experiment; $J_{glucose} = 2.46 \pm 0.16$ μmol/min per g dry mass ($n = 8$) for the phenobarbital-treated group ($P < 0.001$)].

The relationships between some intracellular intermediates and gluconeogenic flux are shown in Fig. 2. The following metabolites were measured: glucose 6-phosphate, fructose 6-phosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, phosphoenolpyruvate and extracellular pyruvate. The relationships between the rate of glucose formation and the concentrations of glucose 6-phosphate, fructose 6-phosphate, dihydroxyacetone phosphate, 3-phosphoglycerate and phosphoenolpyruvate show similar curves for control and phenobarbital-

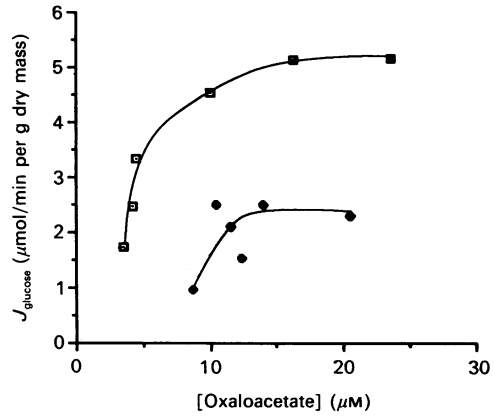

Fig. 3. Relationship between the rate of gluconeogenesis from lactate + pyruvate and the concentration of cytosolic oxaloacetate

Cells from rats treated (◆) or not (⊡) with phenobarbital were perifused as described in Fig. 1. At each steady state, 0.7 ml samples of the cell suspension were removed from the perifusion chamber for rapid separation of mitochondria and cytosol by the digitonin fractionation procedure (see the Materials and methods section). Cytosolic oxaloacetate concentrations were calculated from the cytosolic malate concentrations as described in the Materials and methods section. For lactate concentrations of 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 mmol/l, the lactate/pyruvate ratios were respectively 14, 14, 19, 13, 12 and 9 for the control group and 9, 9, 13, 11, 12 and 9 for the treated group. The glucose concentration was measured in the perifusate. The data are the means of two experiments from two different hepatocyte preparations in each group.

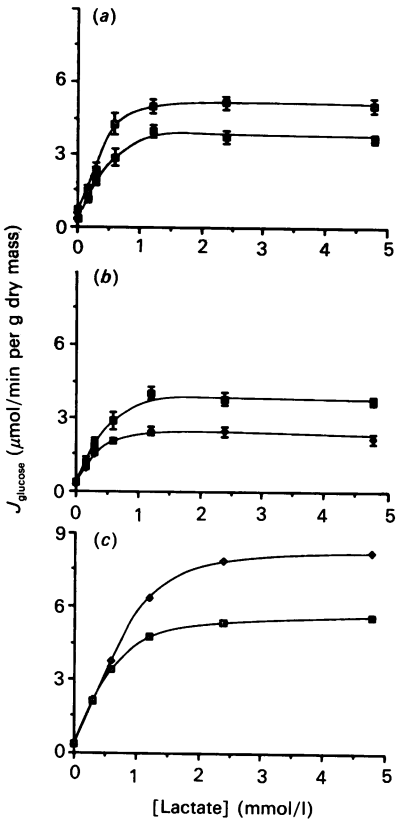

Fig. 4. Effect of phenobarbital treatment on lactate + pyruvate metabolism in perifused hepatocytes in the presence of glucagon

Hepatocytes were isolated and perifused as indicated in Fig. 1: ◆, phenobarbital; ■, phenobarbital and glucagon (0.1 μmol/l); □, control; ◇, control and glucagon (0.1 μmol/l). The rate of glucose formation was calculated from the measured glucose concentration in the perifusate. Results are expressed as means ± S.E.M. (*a*) Comparison between glucose production in cells from control ($n = 5$) and phenobarbital-treated rats stimulated by glucagon addition ($n = 3$). (*b*) Effect of glucagon on glucose production in cells from phenobarbital-treated rats (phenobarbital, $n = 8$; phenobarbital and glucagon, $n = 3$). (*c*) Effect of glucagon on glucose production in control cells (one experiment).

pretreated rats. In contrast, the relationship between the rate of glucose formation and the extracellular pyruvate concentration was different for control and pretreated rats (Fig. 2*f*). These results suggest that the pretreatment does not affect the steps between phosphoenolpyruvate and glucose formation, indicating that the inhibitory effect is probably located somewhere between extracellular pyruvate and phosphoenolpyruvate.

Since it was shown by Groen *et al.* (1983) that pyruvate carboxylase itself determines maximal gluconeogenic flux, we therefore investigated the possibility of a single effect of the chronic phenobarbital treatment on this step. Indeed, in such a hypothesis, we could expect a unique relationship between cytosolic oxaloacetate and glucose production in both control and phenobarbital-treated groups. For this purpose we performed similar experiments in which cytosolic malate was measured in each steady state. As reported previously (Groen *et al.*, 1983; Rigoulet *et al.*, 1987) malate dehydrogenase (EC 1.1.1.37) is, in these conditions, at a near-equilibrium state. This allowed us to calculate cytosolic oxaloacetate from cytosolic malate, taking into account the lactate/pyruvate ratio and the equilibrium constants (lactate dehydrogenase, 1.11×10^{-4}; malate dehydrogenase, 2.78×10^{-5}; Williamson *et al.*, 1967). These results are reported in Fig. 3. It is clear that two different shapes of curves are obtained, i.e. for similar concentrations of cytosolic oxaloacetate, the gluconeogenic flux of the treated group was much smaller than that of the controls. The difference in shape of these two curves excludes a single effect of phenobarbital treatment on pyruvate carboxylase, on transport of pyruvate across the plasma and mitochondrial membranes, and on transport of oxaloacetate to the cytosol. The effect is more likely located at phosphoenolpyruvate carboxykinase, pyruvate kinase or both.

Stimulation of pyruvate kinase flux could explain our results. To examine this hypothesis, we performed experiments in the presence of glucagon (0.1 μmol/l) (Fig. 4) in order to suppress the pyruvate kinase flux. For control cells, we found similar results to those reported by Groen *et al.* (1983), i.e. with glucagon addition, the highest flux of glucose formation was 8.24 μmol/min per g dry mass ($n = 1$), and the percentage stimulation increased progressively with the gluconeogenic flux (Fig. 4*c*). Addition of glucagon also increased glucose production in the phenobarbital group, but the highest rate (3.79 ± 0.25 μmol/min per g dry mass) did not reach that in controls without glucagon (4.99 ± 0.3 μmol/min per g dry mass) (Fig. 4*a*) and was far below the rate after glucagon addition in controls (8.24 μmol/min per g dry mass, $n = 1$) (Fig. 4*c*). It must be pointed out that glucagon addition increased glucose production in phenobarbital-treated cells by about 50% (2.46 to 3.79 μmol/min per g dry mass) (Fig. 4*b*), whereas for the same rate of glucose production in control hepatocytes (2.5 μmol/min per g dry mass), the effect of glucagon is smaller (Groen *et al.*, 1983) or absent (Fig. 4*c*).

We can also postulate an effect of phenobarbital treatment on phosphoenolpyruvate carboxykinase, as already indicated in Fig. 3. In order to test this hypothesis we performed experiments to measure the phosphoenolpyruvate carboxykinase flux control coefficient (Kacser & Burns, 1973; Heinrich & Rapoport, 1974).

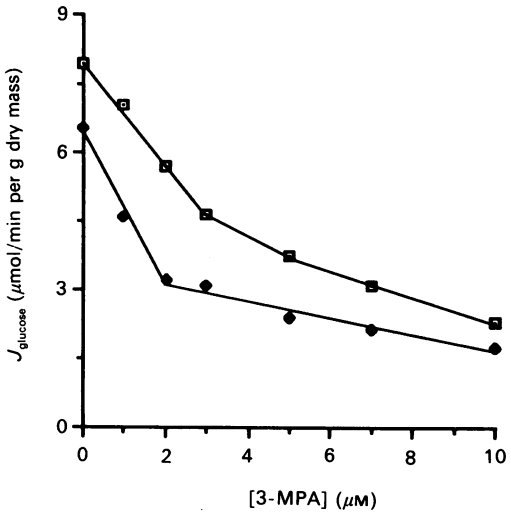

Fig. 5. Inhibition of gluconeogenesis from lactate + pyruvate by 3-MPA

Hepatocytes (220 mg dry mass in 12 ml) from rats treated (◆) or not (□) with phenobarbital were perifused with lactate (4.8 mmol/l) + pyruvate (0.48 mmol/l), oleate (0.1 mmol/l) and glucagon (0.1 μmol/l) and different concentrations of 3-MPA acid. Glucose was measured in the perifusate.

The flux control coefficient of phosphoenolpyruvate carboxykinase can be directly measured by titrating the rate of glucose formation with the non-competitive inhibitor 3-MPA (DiTullio *et al.*, 1974; Jomain-Baum *et al.*, 1976), a procedure also used by Groen and co-workers (Groen *et al.*, 1982*b*, 1986; Rigoulet *et al.*, 1987). Fig. 5 gives the results of this experiment, in which increasing concentrations of the inhibitor were infused in the presence of glucagon and saturating concentrations of lactate plus pyruvate (10:1) in hepatocytes from pretreated or untreated rats. Assuming equilibration of 3-MPA across the plasma membrane, the flux control coefficient of phosphoenolpyruvate carboxykinase can be calculated from the following equation (Groen *et al.*, 1982*b*; Rigoulet *et al.*, 1987):

$$C = -(K_i/J)(dJ/dI)_{I=0}$$

in which J denotes gluconeogenic flux, I is the concentration of the inhibitor and K_i is the inhibition constant (3 μmol/l; Jomain-Baum *et al.*, 1976). This experiment was performed in duplicate (one is shown in Fig. 5). The calculation yielded values for C of 0.32 and 0.34 for the control group and 0.83 and 0.86 for the phenobarbital-treated group. It should be noted that the control values are very close to that previously reported (0.28) by Rigoulet *et al.* (1987). Hence it appears that chronic phenobarbital treatment increases dramatically the flux control coefficient of phosphoenolpyruvate carboxykinase, explaining a major part of the inhibitory effect on gluconeogenesis from lactate plus pyruvate (10:1).

The effect of phenobarbital pretreatment on phosphoenolpyruvate carboxykinase was confirmed by the measurement of phosphoenolpyruvate carboxykinase activity, which was decreased by 50% in the phenobarbital group [7.7 ± 0.5 nmol/min per mg of protein ($n = 4$) versus 16 ± 1.53 nmol/min per mg ($n = 5$) for the control group ($P < 0.002$)].

DISCUSSION

Our results clearly show an inhibition of gluconeogenesis in hepatocytes isolated from rats pretreated for 15 days with phenobarbital (100 mg/kg per day in drinking water). This inhibition of gluconeogenesis *in vitro* was not due to a direct effect of phenobarbital, either used for anaesthesia of the animals or added in the drinking water for the chronic treatment, since Scholz *et al.* (1966) have reported a rapid elimination of phenobarbital during the rat liver perfusion and isolation procedure. Thus the inhibitory effect on gluconeogenesis results mainly from a chronic exposure and not from a direct effect of this drug.

The decrease in the gluconeogenic flux is not due to decreased cell viability in the phenobarbital-treated group during the isolation procedure or closed flask incubation, for two reasons. First, in closed flask incubation and in perifused hepatocytes no difference was found with regard to total nucleotide content: cytosolic and mitochondrial ATP/ADP ratios were slightly but significantly higher in the phenobarbital group (results not shown). Secondly, the inhibitory effect was not found when glycerol was used as gluconeogenic substrate. To our knowledge, this is the first report of inhibition of gluconeogenesis due to chronic phenobarbital in isolated hepatocytes. Scholz *et al.* (1973) reported inhibition of gluconeogenesis in perfused livers isolated from phenobarbital-pretreated rats only when perfused in the presence of aminopyrine (0.2 mmol/l), a substrate for NADPH-utilizing mixed-function oxidation.

Glucose-6-phosphatase, which catalyses the final step in the gluconeogenesis pathway, is a SER-bound enzyme. Changes in its activity following enzyme induction have been reported in the literature, although the results are controversial. Pretreatment with phenobarbital in normal rats, obese or lean female Zucker rats, and lean or obese (*ob/ob*) mice appears to decrease the activity of this enzyme (Orrenius & Ericsson, 1966; Karvonen *et al.*, 1987), whereas in non-insulin-dependent diabetic humans its activity increases (Sotaniemi *et al.*, 1984). In our conditions such an explanation (i.e. modulation of glucose-6-phosphatase activity) is not likely, since the flux control coefficient of this step is very low (less than 2%; Groen *et al.*, 1986; Rigoulet *et al.*, 1987). Moreover, it is clear from our data (Fig. 2*a*) that changes in glucose-6-phosphatase activity do not explain the inhibitory effect of phenobarbital, since we found a single relationship between glucose-6-phosphate concentration and gluconeogenic flux both with and without phenobarbital pretreatment.

Microsomal protein induction due to phenobarbital pretreatment leads to an increase in cytochrome *P*-450 content and to an increase in NADPH-utilizing mixed function oxidation in liver cells. Therefore, when substrates such as aminopyrine are present, the NADPH-utilizing system is enhanced and the NADPH/$NADP^+$ ratio is decreased. Such a decrease in the cellular reducing state has been linked to an effect on carbohydrate metabolism (Junge & Brand, 1975), either by an increase in the pentose pathway or by an increase in malic enzyme flux, both leading to NADPH formation. As reported by Junge & Brand (1975), the flux through the hexose monophosphate shunt can account for all of the reducing equivalents (NADPH) required for enhanced mixed-function oxidation caused by chronic phenobarbital treatment. Thus a decrease in net glucose formation could result from an activation of cycling between glucose 6-phosphate and glyceraldehyde phosphate through the hexose monophosphate shunt. Because there is one decarboxylation step between 6-phosphogluconate and ribulose 6-phosphate, this cycling leads to a decrease in the stoichiometry of conversion of lactate into glucose. This explanation is not relevant for our observations, since we found a single relationship between fructose 6-phosphate (Fig. 2*b*) and gluconeogenic flux, and between dihydroxyacetone phosphate and gluconeogenic flux (Fig. 2*c*), in both controls and phenobarbital-pretreated rats. An alternative explanation was proposed by Scholz *et al.* (1973) via a futile cycle involving

malic enzyme, an NADPH-producing step. Such cycling cannot entirely explain our results, since the inhibition reported by Scholz *et al.* (1973) was found only at saturating lactate concentrations, whereas we report a 35% inhibition at a lactate concentration of only 0.15 mmol/l. Moreover, an effect via a futile cycle involving malic enzyme would lead to a single relationship between cytosolic malate or oxaloacetate and glucose flux regardless of chronic phenobarbital treatment, which it is not the case (Fig. 3). In contrast with experiments reported by Scholz *et al.* (1973), in our perifusion experiments there were no added substrates, such as aminopyrine, that would be metabolized by NADPH-utilizing mixed function oxidases. Hence this explanation cannot entirely account for our results.

The results presented here allow us to propose another explanation involving the pyruvate/phosphoenolpyruvate cycle. In Fig. 2(*e*) it is clearly apparent that a unique relationship was observed between phosphoenolpyruvate and the flux of glucose formation in both groups of hepatocytes. This confirms that in our conditions the main effect of phenobarbital treatment on the gluconeogenic pathway is located upstream from phosphoenolpyruvate. This is in good agreement with our results in closed flask incubations (Table 1), where gluconeogenesis from lactate/pyruvate, alanine or glutamine was decreased by phenobarbital treatment, whereas glycerol metabolism was not affected. Indeed, glycerol metabolism is mainly controlled by the redox state (Williamson *et al.*, 1971; Berry *et al.*, 1973; Leverve *et al.*, 1985, 1986), and phosphoenolpyruvate/pyruvate cycling is not involved in this pathway. As reported by Groen *et al.* (1983, 1986), the main controlling steps of the gluconeogenic pathway from lactate are pyruvate carboxylase, phosphoenolpyruvate carboxykinase and pyruvate kinase, since transport of pyruvate across the plasma membrane (Denton & Halestrap, 1979) and the mitochondrial membrane (Groen *et al.*, 1983) and the transport of oxaloacetate (in the form of aspartate) to the cytosol (Williamson, 1976) have high capacities. Moreover, the two distinct relationships between cytosolic oxaloacetate concentration and glucose flux (Fig. 3) in the two groups of cells exclude a single effect on either pyruvate transport, or pyruvate carboxylase or oxaloacetate transport. Therefore inhibition of gluconeogenesis must be at least partly the consequence of an effect on phosphoenolpyruvate carboxykinase, pyruvate kinase or both.

The flux through pyruvate kinase, by creating a futile cycle between pyruvate and phosphoenolpyruvate, is largely involved in the control of the gluconeogenic flux (Groen *et al.*, 1983, 1986). The percentage stimulation by glucagon in the phenobarbital-treated group was higher than that in the control cells at a similar glucose flux, as reported by Groen *et al.* (1986) and confirmed here. Hence it could be proposed that phenobarbital treatment enhances the pyruvate kinase flux, leading to a greater inhibitory effect of glucagon on this step. This result suggests some effect of chronic phenobarbital treatment which activates phosphoenolpyruvate/pyruvate cycling. However, a pure effect via the pyruvate kinase step cannot alone explain the inhibitory effect of phenobarbital since, as reported in Fig. 1, in cells from phenobarbital-treated animals the flux in the presence of glucagon does not reach that in controls and is far below the glucagon-stimulated flux reported in the literature (Groen *et al.*, 1983; Rigoulet *et al.*, 1987) and by us.

It is well known that the phosphoenolpyruvate carboxykinase activity changes rapidly with nutritional and hormonal status. In the livers of adult rats, starvation and diabetes increase this activity, whereas refeeding carbohydrate to starved rats, insulin treatment of diabetic rats and changes in nutrition during the suckling–weaning transition have the opposite effect (Kida *et al.*, 1980; Cimbala *et al.*, 1982; Beale *et al.*, 1982, 1984; Lyonnet *et al.*, 1988). In accordance with the high amount of phosphoenolpyruvate carboxykinase present in the fasted state, the gluconeogenic flux is predominantly controlled by other steps, i.e. pyruvate carboxylase and pyruvate kinase. On the contrary, in the fed state a dramatic decrease in phosphoenolpyruvate carboxykinase activity leads to a shift in the flux control structure of this pathway toward phosphoenolpyruvate carboxykinase, resulting in a high flux control coefficient for this step (Groen & Tager, 1988).

Our results show that the flux control coefficient of phosphoenolpyruvate carboxykinase is much higher in the phenobarbital-treated cells (0.80) than in controls (0.30), in agreement with the observation that the enzyme activity decreases by 50% on phenobarbital treatment. The chronic effect of phenobarbital on the gluconeogenic pathway could be viewed as a shift from the 'fasted state' to a 'fed state', even in 24 h-fasted animals.

It should be noted that for each steady state cytosolic oxaloacetate concentrations are in the same range in both the control and phenobarbital groups. This is rather important since, as pointed out by Rigoulet *et al.* (1987), the elasticity coefficient of pyruvate carboxylase towards its product oxaloacetate plays a crucial role, because it primarily determines the control coefficient of pyruvate carboxylase, and thus the flux to the overall pathway. From this viewpoint the effect on phosphoenolpyruvate carboxykinase of chronic treatment leads to a relative increase in oxaloacetate concentrations, which decrease the rate of flux through pyruvate carboxylase and, hence, glucose formation.

The question arises as to the nature of the link between chronic phenobarbital treatment, microsomal protein induction and the resulting effect on the phosphoenolpyruvate carboxykinase step. It could be proposed that chronic phenobarbital treatment directly or indirectly affects the phosphoenolpyruvate carboxykinase concentration, by decreasing the synthesis or by increasing the breakdown of this protein. Indeed, the changes with nutritional and hormonal status in phosphoenolpyruvate carboxykinase activity are accompanied by a variation in the concentration of phosphoenolpyruvate carboxykinase mRNA (Cimbala *et al.*, 1982; Beale *et al.*, 1982, 1984; Lyonnet *et al.*, 1988). Insulin and glucagon regulate liver phosphoenolpyruvate carboxykinase by modifying its gene transcription (Lamers *et al.*, 1982; Granner & Andreone, 1985). We suggest that phenobarbital treatment decreases phosphoenolpyruvate carboxykinase gene transcription by affecting the insulin/glucagon ratio.

We thank Claudette Guidicelli (C.E.N.G., Grenoble) for determination of cytochrome *P*-450, Betty Gouhot (C.N.R.S., UPR 1511 Meudon Bellevue) for determination of phosphoenolpyruvate carboxykinase activity, Michel Rigoulet and Christiane Keriel for helpful discussion, Nicolas Wiernsperger and Philippe André (Lipha Laboratory), and the Joseph Fourier University for financial support.

REFERENCES

Beale, E. G., Hartley, J. L. & Granner, D. K. (1982) J. Biol. Chem. **257**, 2022–2028

Beale, E. G., Andreone, T., Koch, S., Granner, M. & Granner, D. K. (1984) Diabetes **33**, 328–332

Bergmeyer, H. U. (ed.) (1970) Methoden der Enzymatischen Analyse, 2nd edn., Verlag Chemie, Weinheim

Berry, M. N. & Friend, D. S. (1969) J. Cell Biol. **43**, 506–520

Berry, M. N., Lund, P. & Werner, H. V. (1973) Eur. J. Biochem. **33**, 407–417

Best, J. D., Judzewitsch, R. G., Pfeifer, M. A., Beard, J. C. & Halter, J. B. (1982) Diabetes **31**, 333–338

Chang, H. C. & Lane, M. D. (1966) J. Biol. Chem. **241**, 2413–2420

Cimbala, M. A., Lamers, W. H., Nelson, K., Monahan, J. E., Yoo-Warren, H. & Hanson, R. W. (1982) J. Biol. Chem. **257**, 7629–7636

Conney, A. H. (1967) Pharmacol. Rev. **19**, 317–365
Denton, R. M. & Halestrap, A. P. (1979) Essays Biochem. **15**, 37–77
DiTullio, N. W., Berkoff, C. E., Blank, K., Kostos, V., Stack, E. J. & Saunders, H. L. (1974) Biochem. J. **138**, 387–394
Granner, D. K. & Andreone, T. L. (1985) Diabetes/Metab. Rev. **1**, 139–170
Groen, A. K. & Tager, J. M. (1988) in Integration of Mitochondrial Function (Lemasters, J. J., Hackenbrock, C. R., Thurman, R. G. & Westerhoff, H. V., eds.), pp. 245–260, Plenum Press, New York and London
Groen, A. K., Sips, H. J., Vervoorn, R. C. & Tager, J. M. (1982*a*) Eur. J. Biochem. **122**, 87–93
Groen, A. K., van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, T. P. M. & Tager, J. M. (1982*b*) in Metabolic Compartmentation (Sies, H., ed.), pp. 9–37, Academic Press, New York
Groen, A. K., Vervoorn, R. C., van der Meer, R. & Tager, J. M. (1983) J. Biol. Chem. **258**, 14346–14353
Groen, A. K., Van Roermund, C. W. T., Vervoorn, R. C. & Tager, J. M. (1986) Biochem. J. **237**, 379–389
Heinrich, R. & Rapoport, T. A. (1974) Eur. J. Biochem. **42**, 89–95
Jomain-Baum, M., Schramm, V. L. & Hanson, R. W. (1976) J. Biol. Chem. **251**, 37–44
Junge, O. & Brand, K. (1975) Arch. Biochem. Biophys. **171**, 398–406
Kacser, H. & Burns, J. A. (1973) in Rate Control of Biological Processes (Davies, D. D., ed.), p. 65, Cambridge University Press, Cambridge
Karvonen, I., Stengärd, J. H., Saarni, H. U., Stenbäck, F. & Sotaniemi, E. A. (1987) Diabetes Res. **4**, 195–200
Karvonen, I., Stengärd, J. H., Huupponen, R., Stenbäck, F. G. & Sotaniemi, E. A. (1989) Diabetes Res. **10**, 85–92
Kida, K., Nishio, T., Yokosawa, T., Nagai, K., Matsuda, H. & Nagakawa, H. (1980) J. Biochem. (Tokyo) **88**, 1009–1013
Lamers, W. H., Hanson, R. W., Meisner, H. M. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 5137–5141
Lathela, J. T., Särkkä, P. & Sotaniemi, E. A. (1984) Res. Commun. Chem. Pathol. Pharmacol. **44**, 215–226
Lathela, J. T., Arranto, A. J. & Sotaniemi, E. A. (1985) Diabetes **34**, 911–916
Lathela, J. T., Arranto, A. J., Stenbäck, F. & Sotaniemi, E. A. (1986) Scand. J. Gastroenterol. **21**, 737–743
Leverve, X. M., Groen, A. K., Verhoeven, A. J. & Tager, J. M. (1985) FEBS Lett. **181**, 43–46
Leverve, X. M., Verhoeven, A. J., Groen, A. K., Meijer, A. J. & Tager, J. M. (1986) Eur. J. Biochem. **155**, 551–556
Lyonnet, S., Coupe, C., Girard, J., Kahn, A. & Munnich, A. (1988) J. Clin. Invest. **81**, 1682–1689
Marshall, W. J. & McLean, A. E. M. (1969) Biochem. Pharmacol. **18**, 153–157
Omura, T. & Sato, R. (1964) J. Biol. Chem. **239**, 2370–2378
Orrenius, S. & Ericsson, J. L. E. (1966) J. Cell Biol. **31**, 243–256
Orrenius, S., Ericsson, J. L. E. & Ernester, L. (1965) J. Cell Biol. **25**, 627–639
Remmer, H. (1972) Eur. J. Clin. Pharmacol. **5**, 116–136
Rigoulet, M., Leverve, X. M., Plomp, P. J. A. & Meijer, A. J. (1987) Biochem. J. **245**, 661–668
Scholz, R., Schwarz, R. & Bücher, Th. (1966) Z. Klin. Chem. **4**, 179–189
Scholz, R., Hansen, W. & Thurman, R. G. (1973) Eur. J. Biochem. **38**, 64–72
Schulte-Hermann, R. (1974) CRC Crit. Rev. Toxicol. **3**, 97–158
Sotaniemi, E. A., Stengärd, J. H., Saarni, H. U., Arranto, A. J., Keinänen, K., Kerola, T. & Sutinen, S. (1984) Acta Med. Scand. **215**, 323–331
van der Meer, R. & Tager, J. M. (1976) FEBS Lett. **67**, 36–40
Williamson, D. H., Lund, P. & Krebs, H. A. (1967) Biochem. J. **103**, 514–527
Williamson, J. R. (1976) in Gluconeogenesis (Hanson, R. W. & Mehlman, M. A., eds.), pp. 165–220, Wiley, New York
Williamson, J. R., Jacob, A. & Refino, C. (1971) J. Biol. Chem. **246**, 7632–7641
Zuurendonk, P. F. & Tager, J. M. (1974) Biochim. Biophys. Acta **333**, 393–399

Received 7 February 1991/3 July 1991; accepted 18 July 1991