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Control of glucuronidation during hypoxia

Limitation by UDP-glucose pyrophosphorylase

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The regulation of glucuronidation during hypoxia was studied in isolated hepatocytes by analysing the dependence of acetaminophen glucuronidation rate on the intracellular concentrations of UTP, glucose 1-phosphate, UDP-glucose and UDP-glucuronic acid. The steady-state concentrations of these metabolites in cells from fed and starved rats were altered by exposure to various hypoxic O_2 concentrations and by adding exogenous glucose. Changes in glucuronidation rate under all conditions were explained in terms of the concentrations of the substrates for UDP-glucose pyrophosphorylase, i.e. UTP and glucose 1-phosphate. Steady-state rates for the UDP-glucose pyrophosphorylase reaction, calculated by using published kinetic constants and measured glucose 1-phosphate and UTP concentrations, were in agreement with the measured glucuronidation rates. Thus the UDP-glucose pyrophosphorylase reaction is the key regulatory site for drug glucuronidation during hypoxia. Control at this site indicates that glucuronidation *in vivo* may be generally depressed in pathological conditions involving hypoxia and energy (calorie) malnutrition.

Glucuronidation is an important metabolic pathway in which endogenous compounds (e.g. bilirubin) and foreign compounds (e.g. acetaminophen) are converted into more polar metabolites for excretion. In recent studies we found that this pathway is sensitive to O_2 concentration in isolated hepatocytes (Aw & Jones, 1982*a*) and *in vivo* in chronically hypoxic patients (Kaplan *et al.*, 1983). In isolated hepatocytes, glucuronidation rate was altered by several factors which changed the cellular ATP/ADP ratio, such as exposure to hypoxia or to respiratory inhibitors. However, under low O_2 concentration, the rate of glucuronidation was stimulated by addition of exogenous glucose under conditions where the glucose had no detectable effect on the ATP/ADP ratio. These results suggest that stimulation of glycolysis during hypoxia may result in limited availability of the glucose moiety for synthesis of UDP-GlcA.

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Abbreviations used: acetaminophen (paracetamol), 4'-hydroxyacetanilide; UDP-GlcA, UDP-glucuronic acid; Glc1*P*, glucose 1-phosphate; UDP-Glc, UDP-glucose; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; h.p.l.c., high-pressure liquid chromatography.

Several studies have shown that drug-glucuronidation rate is dependent on the supply of UDP-GlcA (Moldéus *et al.*, 1979; Singh & Schwarz, 1981; Thurman *et al.*, 1981). UDP-GlcA is synthesized from UTP and Glc1*P* in two sequential reactions catalysed by UDP-Glc pyrophosphorylase and UDP-Glc dehydrogenase (Oliver, 1961; Albrecht *et al.*, 1966; Dutton, 1980). Because of this multi-step sequence, a variety of metabolic conditions can potentially affect the overall process.

In the present study we examined the rate of glucuronide formation in hepatocytes as a function of the cellular concentrations of UDP-GlcA, UDP-Glc, Glc1*P* and UTP under conditions where these cellular metabolite concentrations were modified by starvation or addition of exogenous glucose. The results show that rate limitation occurs as a consequence of substrate limitation for the UDP-Glc pyrophosphorylase reaction.

Materials and methods

Cell preparation and incubations

Hepatocytes were isolated from fed and 24h-starved male white rats (Charles Rivers; 180–250g) as described by Moldéus *et al.* (1978*b*). Cells

were metabolically intact as ascertained by several criteria (Moldéus *et al.*, 1978*b*; Jones & Mason, 1978), and routinely 95–99% excluded 0.2% Trypan Blue. The cells maintained viability under the conditions of incubations. Incubations (10^6 cells/ml) were performed with Krebs–Henseleit (1932) buffer containing 12.5mM-Hepes, pH 7.4, in rotating round-bottom flasks at 37°C (Aw & Jones, 1982*a*; Moldéus *et al.*, 1978*b*). The acetaminophen concentration was 5mM. O_2-dependences of glucuronide formation and cellular metabolite concentrations were studied under various steady-state O_2 concentrations, as previously described (Aw & Jones, 1982*a*). Glucuronidation rates are given for 30min incubations and were linear for at least 45min under all the conditions used. 'Steady-state' metabolite concentrations are given for 30min incubations and were essentially identical with 15min values. Identical incubations in SO_4^{2-}-free medium showed that glucuronidation rates were unaffected by changes in sulphation rates under these conditions.

Analytical methods

H.p.l.c. Protein was removed from cell incubations by addition of 0.5ml of 3M-$HClO_4$ (per ml of incubation mixture), followed by centrifugation. The glucuronide conjugate of acetaminophen was quantified by h.p.l.c. (Aw & Jones, 1982*a*). Cellular ATP and ADP were also measured by h.p.l.c. (Jones, 1981*a*).

Metabolite measurements. Incubations (10^6 cells/ml) were stopped by heating the cell mixtures for 2min in a boiling-water bath and then by cooling immediately on ice. Denatured proteins were removed by centrifugation and the supernatants were filtered through 0.45μm-pore-size Millipore filters (Millipore Corp., Bedford, MA, U.S.A.). Enzyme-coupled spectrophotometric assays were performed for UTP (Keppler *et al.*, 1974), Glc1*P* (Bergmeyer & Michal, 1974) and UDP-Glc (Zalitis *et al.*, 1972). UDP-GlcA was measured by h.p.l.c. (Aw & Jones, 1982*b*).

Materials

Collagenase (Type IV) and acetaminophen were purchased from Sigma. All other chemicals used were of reagent grade and were purchased locally. Solvents used for h.p.l.c. were of 'h.p.l.c.' grade and were purchased from Fisher (Atlanta, GA, U.S.A.).

Results

The drug-glucuronidation rate in hepatocytes from starved animals was substantially more sensitive to hypoxia than previously found for cells from fed animals (Fig. 1). Half-maximal activities (P_{50} values) were at 11.5 ± 0.6 ($n = 21$) and 6.6 ± 1.0μM-O_2 ($n = 5$) respectively. Addition of 10mM-glucose stimulated the rate of glucuronidation in cells from both starved and fed animals and lowered the P_{50} values to 7.6 and 2.4μM respectively. The fact that addition of glucose did not eliminate the difference between glucuronidation rates for 'starved' and 'fed' cells suggests that there is a difference in the relative contributions of different pathways in the starved (glycogen-depleted) and fed cells which alters the concentrations of phosphorylated glucose intermediates (see below). For instance, there is likely to be a different rate of glycogen synthesis in the starved cells than in the fed cells, and this enhanced rate of removal would decrease the steady-state concentrations of Glc1*P* and UDP-Glc. These different conditions (starvation, glucose addition and hypoxia) gave a 50-fold variation in rate due to metabolic conditions that can occur *in vivo*. They therefore provide a suitable model to analyse the control of glucuronidation during hypoxia.

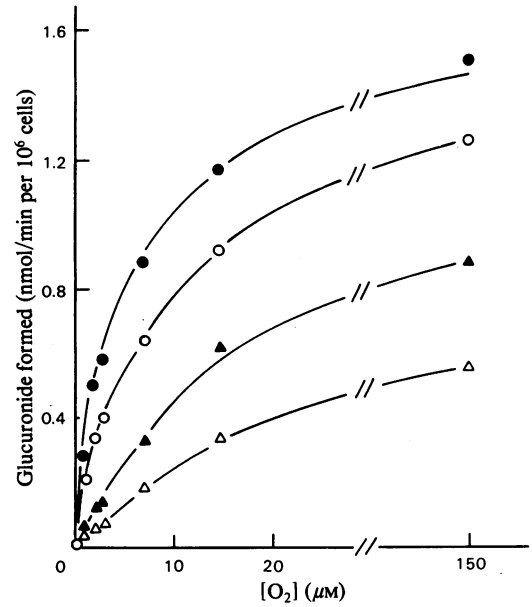

Fig. 1. *O_2-dependence of glucuronidation in fed and starved hepatocytes*

Hepatocytes (10^6 cells/ml) were preincubated under varied steady-state O_2 concentrations for 30min and acetaminophen (5mM) was added. Incubations were carried out for 30min in the presence or absence of 10mM-glucose and analysed as described in the text. Open symbols represent control cells from fed (○, $n = 21$) or starved (△, $n = 5$) rats and closed symbols represent glucose-supplemented-cells from fed (●, $n = 11$) or starved (▲, $n = 5$) rats.

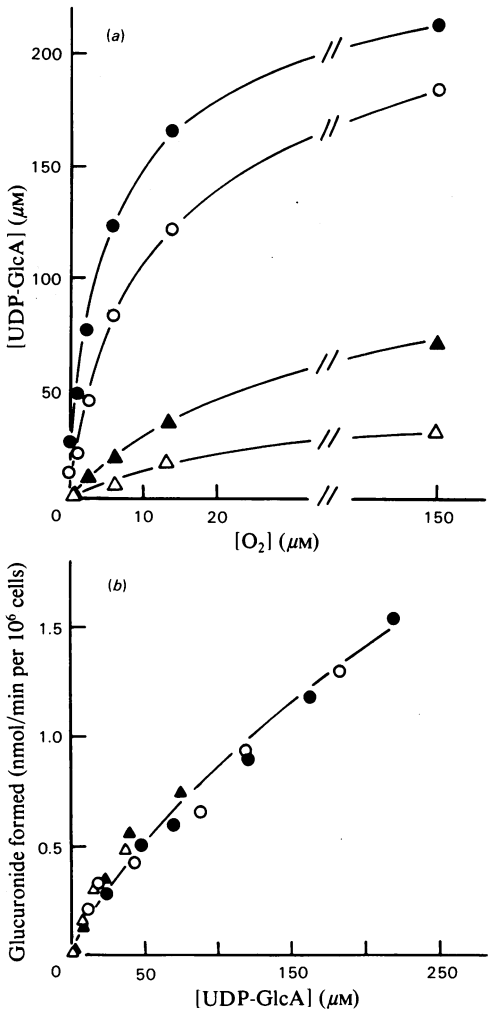

Fig. 2. *Dependence of UDP-GlcA on O_2 concentration and of glucuronidation rate on UDP-GlcA concentration*
Hepatocytes (10^6 cells/ml) from fed (circles) and 24h starved (triangles) rats were incubated under varied steady-state O_2 concentrations in the presence of 5mM-acetaminophen and with (●, ▲) or without (○, △) exogenous glucose (10mM). Incubations were stopped by heating the cell mixtures, and denatured proteins were removed by centrifugation. UDP-GlcA was measured in the supernatants by h.p.l.c. (Aw & Jones, 1982*b*). (*a*) O_2-dependence of UDP-GlcA concentration; (*b*) glucuronidation rate as a function of UDP-GlcA concentration. Rates of glucuronide formation from Fig. 1 were replotted as a function of cellular UDP-GlcA concentration.

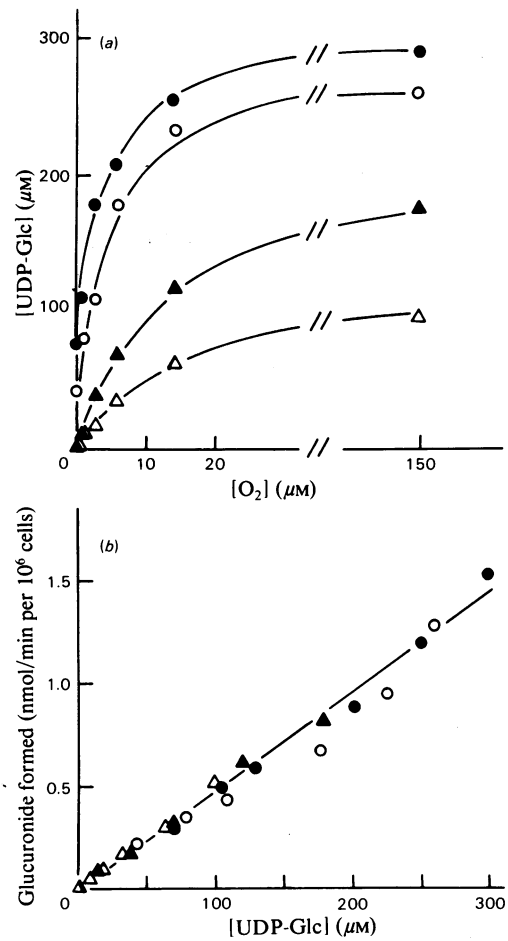

Fig. 3. *Dependence of glucuronidation rate on UDP-Glc concentration in cells*
Hepatocytes (10^6 cells/ml) from fed (circles) and 24h starved (triangles) rats were incubated under various steady-state O_2 concentrations in the presence of 5mM-acetaminophen and with (●, ▲) or without (○, △) 10mM-glucose and assayed as described in the text. (*a*) UDP-Glc as a function of O_2 concentration; (*b*) glucuronide formation as a function of cellular UDP-Glc concentration. Glucuronidation rates from Fig. 1 were expressed as a function of UDP-Glc concentration.

The response of cellular UDP-GlcA concentrations (Fig. 2*a*) was very similar to that seen for the glucuronidation rate, and a direct comparison of the two revealed that the glucuronidation rate was proportional to the UDP-GlcA concentration (Fig. 2*b*). Consequently, the major limiting factor under these conditions appears to be UDP-GlcA supply.

UDP-GlcA is synthesized from UDP-Glc by two successive NAD^+-dependent dehydrogenations. Since the NADH/NAD^+ ratio is known to increase during hypoxia (Chance, 1976), the O_2-dependence of pyridine nucleotides was examined. The change in oxidation–reduction state occurred over the same O_2 concentration range as did the change in glucuronidation rate ($P_{50} = 4.6\,\mu$M-O_2), but was unaffected by starvation or by the addition of 10mM-glucose (results not shown). Therefore the differences in UDP-GlcA concentrations (Fig. 2*a*) and glucuronidation rates (Fig. 1) are not caused by

differences in O_2-sensitivity of the NADH/NAD^+ ratio with starved cells or with added glucose.

Measurement of cellular UDP-Glc concentration under the same conditions (Fig. 3*a*) showed that the glucuronidation rate was also proportional to the cellular UDP-Glc concentration (Fig. 3*b*). Therefore the factors affecting the availability of UDP-Glc determine the overall glucuronidation rate. UDP-Glc is synthesized from UTP and Glc1*P* by the cytosolic transferase UDP-Glc pyrophosphorylase. Measurements of cellular UTP and Glc1*P* concentrations (Figs. 4 and 5*a*) and comparison with measured glucuronidation rates (Figs. 4*b* and 5*b*) revealed that the rate could not be expressed simply as a function of one of these components. Since the apparent-K_m values for UDP-Glc pyrophosphorylase are known, we examined whether the observed steady-state rates of glucuronidation could be calculated from the measured metabolite concentrations (Fig. 6). The reaction was modelled by assuming independent binding of UTP and Glc1*P* with estimated K_m

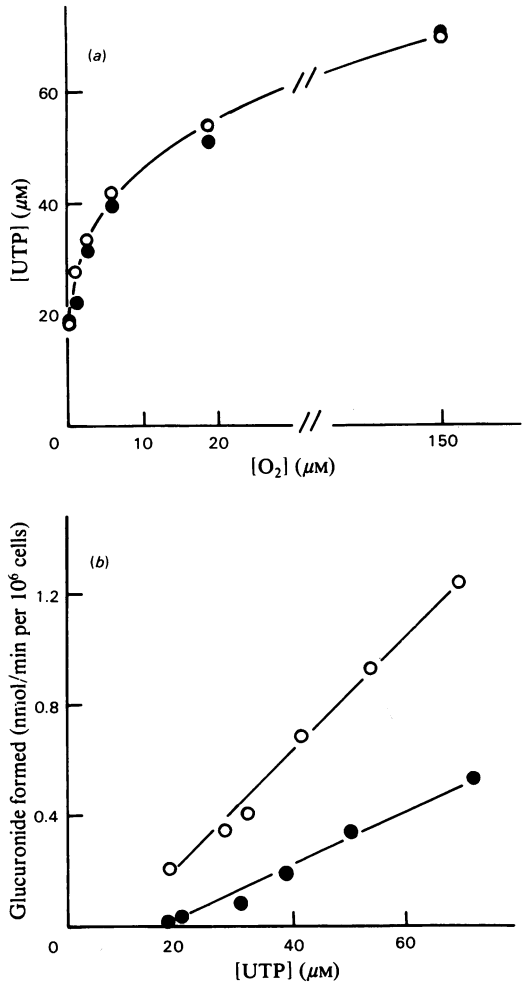

Fig. 4. *Dependence of glucuronidation rate on cellular UTP concentration*

The O_2-dependence of cellular UTP concentration was studied in cells from fed (○) and starved (●) rats as described in Fig. 1. (*a*) UTP concentration in cells was measured as described in the text and is expressed as a function of O_2 concentration; (*b*) glucuronidation rates from Fig. 1 were replotted as a function of cellular UTP concentrations.

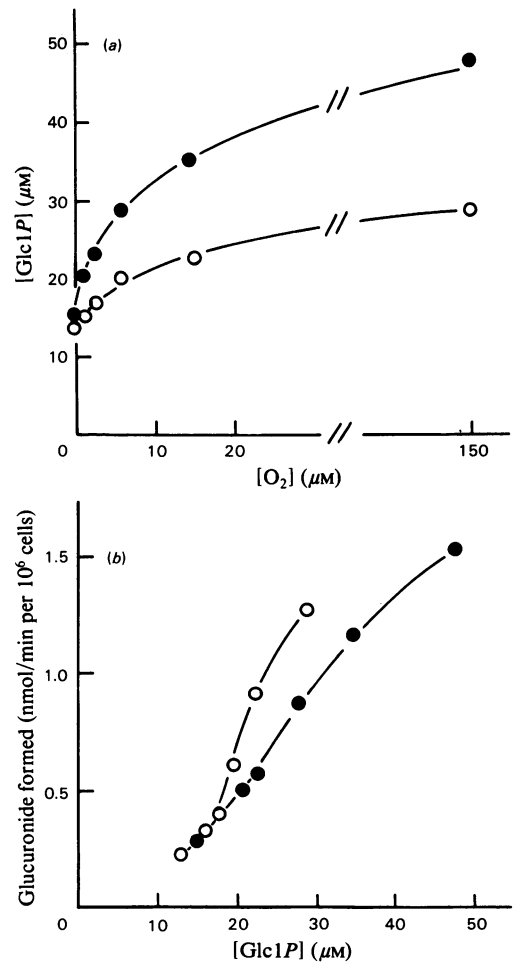

Fig. 5. *Dependence of glucuronidation rate on Glc1P concentration*

Hepatocytes (10^6 cells/ml) from fed rats were incubated with 5mM-acetaminophen and with (●) or without (○) 10mM-glucose, and assayed for Glc1*P* as described in the text. (*a*) Glc1*P* as a function of O_2 concentration; (*b*) glucuronidation rate as a function of Glc1*P* concentration. Rates of conjugation in fed cells from Fig. 1 were replotted as a function of Glc1*P* concentration.

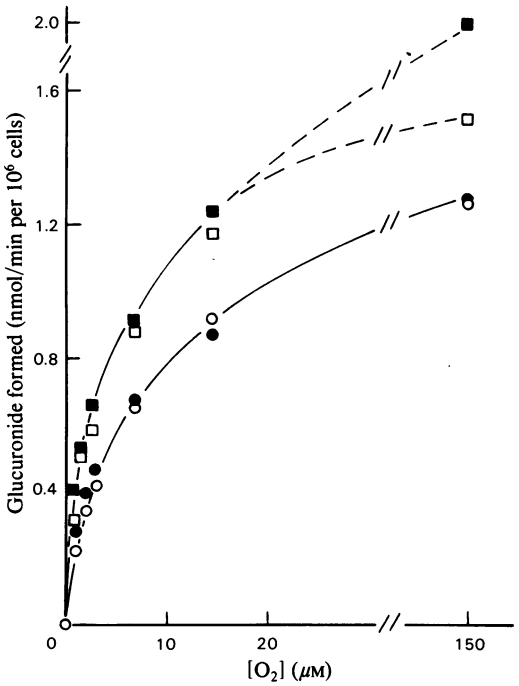

Fig. 6. *Comparison of measured and predicted rates of glucuronidation*

Open symbols represent measured glucuronidation rates for cells from control animals in the absence (○) and presence (□) of 10mM-glucose as in Fig. 1. Closed symbols represent corresponding calculated rates assuming that the synthesis of UDP-Glc is rate-limiting. The rate of the UDP-Glc pyrophosphorylase reaction was calculated by using the equation:

$$v = \frac{V_{max.}}{\left(1+\frac{K_{UTP}}{[UTP]}\right)\left(1+\frac{K_{Glc1P}}{[Glc1P]}\right)}$$

where K_{UTP} was 125μM and K_{Glc1P} was 180μM (Sadurska & Szymczyk, 1978). $V_{max.}$ was estimated to be 26.5nmol/min per 10^6 cells, but this value cannot be accurately assessed under the given conditions and does not significantly affect the modelling because both substrate concentrations are significantly below the K_m values.

values for the rat liver enzyme of 125μM and 180μM respectively (Sadurska & Szymczyk, 1978). The calculated results are in good agreement with the measured rates, except at 150μM-O_2 in the presence of exogenous glucose. Under these conditions, the actual rate was considerably below the predicted rate, indicating that another factor, such as product inhibition by UDP-Glc (Albrecht *et al.*, 1966; Sadurska & Szymczyk, 1978) also affects the rate.

Discussion

Our previous studies showed that glucuronidation is sensitive to O_2 concentration, even though O_2 is not required directly as a substrate for the pathway (Aw & Jones, 1982*a*). The present studies show that these changes in rate can be explained in terms of changes in the concentrations of metabolic intermediates, specifically Glc1*P* and UTP. The availability of these substrates for UDP-Glc pyrophosphorylase determines the rate of UDP-Glc synthesis. The conversion of UDP-Glc into UDP-GlcA and the transfer of the glucuronosyl moiety to acetaminophen are then established at the same steady-state rates as functions of their concentrations. The measured K_m values of UDP-glucuronosyltransferases for UDP-GlcA are consistent with this interpretation, since they are high (0.16–1.3mM) (Winsnes, 1972; Bock *et al.*, 1973) relative to the intracellular UDP-GlcA concentrations (up to 200μM).

Previous studies suggested that glucuronidation may be influenced by the availability of NAD^+ during hypoxia (Moldéus *et al.*, 1978*a*; Jones, 1981*b*). However, the O_2-dependence of nicotinamide nucleotide oxidation was unaffected by starvation or addition of glucose, and therefore the change in glucuronidation rate appears to be independent of the change in oxidation state of nicotinamide nucleotides. Thus control of glucuronidation during hypoxia is distinct from the control during ethanol metabolism, since in the latter, glucuronidation rate is determined in part by the oxidation state of nicotinamide nucleotides (Aw & Jones, 1983). Presumably the metabolism of ethanol causes a more significant change in the cytosolic NAD^+/NADH ratio than does hypoxia. Thus, during hypoxia in the absence of ethanol, the supply of Glc1*P* and UTP becomes limiting before NAD^+/NADH becomes limiting.

Recent studies on the disposition of acetaminophen in hypoxic patients demonstrate that inhibition of glucuronidation during hypoxia occurs *in vivo* (Kaplan *et al.*, 1983). The urinary recoveries of the glucuronide conjugate were significantly elevated on O_2 administration to a population with chronic bronchitis and emphysema. Thus the observations concerning the control of glucuronidation during hypoxia have direct practical applications. Drugs metabolized principally by glucuronidation can be expected to be cleared more slowly during hypoxia. Because glucose supply further limits glucuronidation, the process may be further impaired by energy (calorie)-poor diets, and therefore proper dietary management is critical.

In conclusion, the results show that the limitation of glucose availability (as Glc1*P*) and UTP

concentration are important in control of the rate of glucuronidation during hypoxia. The interaction of glucose availability and UTP supply indicates that energy deficiency may be an important factor in pathogenic processes due to hypoxia. Because the combined effect of these processes results in O_2-sensitivity at rather high O_2 concentrations (25 μM), perturbations in this type of process may occur under mildly hypoxic conditions and may therefore be a sensitive biochemical index of hypoxia.

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