Zonation of gluconeogenesis, ketogenesis and intracellular pH in livers from normal and diabetic ketoacidotic rats: evidence for intralobular redistribution of metabolic events in ketoacidosis

Shamus P. BURNS^{*1}, Robert D. COHEN^{*}, Richard A. ILES^{*}, Rosemary A. BAILEY[†], Mina DESAI[‡], Jocelyn P. GERMAIN[§] and Thomas C. H. GOING^{*}

^{*}Medical Unit (Whitechapel), St Bartholomew's and the Royal London Hospital School of Medicine and Dentistry, London E1 1BB, U.K., [†]School of Mathematical Sciences, Queen Mary and Westfield College, London E1 4NS, U.K., [‡]Department of Clinical Biochemistry, University of Cambridge Clinical School, Addenbrooke's Hospital, Cambridge CB2 2QR, U.K., and [§]Department of Oral Pathology, St Bartholomew's and the Royal London Hospital School of Medicine and Dentistry, London E1 1BB, U.K.

The intralobular distribution of metabolism was examined in the livers from rats with severe diabetic ketoacidosis (DKA), perfused at pH 6.8, and compared with that in livers from normal starved animals perfused at either pH 7.4 or 6.8. With lactate and palmitate as substrates, the perivenous uptake of periportal synthesized glucose seen in normal livers at pH 7.4 was abolished during DKA; indeed, gluconeogenesis was most active in the perivenous region. Whereas in normal livers perfused at pH 7.4 the periportal region showed a markedly elevated intracellular pH (pH_i) compared with the perivenous zone, this distribution of pH_i was reversed in DKA, with an intermediate distribution in normal livers perfused at pH 6.8. 3-Hydroxybutyrate was generated throughout the lobule. Some acetoacetate generated peri-

portally was converted to 3-hydroxybutyrate more perivenously. A steep gradient of oxygen uptake along the radius of the lobule was apparent in all three groups; oxygen uptake was greatly decreased perivenously despite adequate oxygen supply. These findings are consistent with our previous observations of the lobular co-location of high pH_i and gluconeogenesis, and might offer an explanation of how high gluconeogenic rates can continue in spite of severe systemic acidosis in DKA. The findings provide direct evidence for a marked redistribution of intralobular metabolism in DKA.

Key words: diabetic ketoacidosis, hepatocyte heterogeneity, streptozotocin.

INTRODUCTION

In diabetic ketoacidosis (DKA) an increased hepatic output of glucose is the major contributor to hyperglycaemia [1]. An apparent anomaly exists in that systemic acidosis can be severe and hepatic intracellular acidosis is known to inhibit gluconeogenesis [2–6]. We have previously demonstrated that perfused livers of diabetic ketoacidotic rats fail to show the inhibition of gluconeogenesis from lactate that is seen in livers from normal animals when perfused at pH 6.8 [7]. It has, however, been shown that rat hepatic intracellular pH (pH_i), both *in vivo* and in perfused liver, falls very little in DKA in comparison with other metabolic acidoses [7]; we have suggested that this protection of pH_i is the reason that high rates of hepatic glucose output are achievable in DKA notwithstanding the systemic acidosis [7]. These observations of pH_i and gluconeogenesis were made with ³¹P-NMR in whole liver, both *in vivo* and perfused, and took no account of the possible variation of pH_i or gluconeogenesis in different zones of the hepatic lobule.

We have described a method for the detailed mapping of function, metabolite concentration and enzyme activity along the radius of the hepatic lobule in the perfused liver that is not dependent on the preparation of isolated hepatocytes from the various zones [8]. The method retains the anatomical and vascular relationships of the hepatocytes and is suitable for investigations *ex vivo* with NMR. With this method we have shown that, in livers from normal starved rats, perfused with lactate as the sole

substrate, gluconeogenesis is confined to the periportal cells, whereas perivenous cells normally take up some glucose that has been produced periportal [9]. Hepatic glucose production is therefore a function of periportal hepatocytes and is not normally a characteristic of the perivenous zone of the liver lobule. Furthermore, in normal livers the periportal cells have a higher pH_i than those in the perivenous zone [8] when lactate is the sole substrate; this is attributable to proton consumption during gluconeogenesis from the lactate ion.

We have determined the distribution of hepatic pH_i in a rat model of DKA, and the sites of ketone body production, glucose synthesis and utilization, and oxygen consumption. The current studies characterize the hepatic zonal distribution of these metabolic events and their changes in a model of severe DKA. The information obtained allowed us to examine whether, at the detailed intralobular level, there was a relationship between glucose metabolism and pH_i .

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: streptozotocin, L-(+)-lactate and sodium palmitate were from Sigma-Aldrich Ltd. (Poole, Dorset, U.K.); BSA (fraction V), 3-hydroxybutyrate dehydrogenase and digitonin were from

Abbreviations used: DKA, diabetic ketoacidosis; pH_i , intracellular pH; FVR, fractional volume remaining.

¹ To whom correspondence should be addressed (e-mail s.p.burns@mds.qmw.ac.uk).

Boehringer Mannheim, Lewes, East Sussex, U.K.). All other reagents were of analytical grade.

Procedures

Male Wistar rats (260–320 g), fasted for 48 h, were used. In some, DKA was induced by intrajugular injection of 120 mg/kg streptozotocin, as described previously [7], at the start of the fast. At 48 h, animals were anaesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally. Immediately before liver perfusion, blood was obtained from the inferior vena cava (distal to the renal veins) for the determination of metabolites, pH, p_{CO_2} and p_{O_2} . For metabolites, 1 ml of blood was added to 2 ml of 20% (v/v) HClO_4 and stored at -70°C . Among those animals with DKA, only those with a vena caval blood pH less than or equal to 7.0 were selected for study.

Isolated liver perfusions were set up as described previously [10]. Three groups of perfused livers were studied, as follows: group 1, livers from normal rats, perfused at pH 7.4; group 2, livers from normal rats, perfused at pH 6.8; group 3, livers from DKA rats, perfused at pH 6.8. The flow rate was 12.5 ml/min per 100 g body weight, which ensured that the hepatic venous p_{O_2} was always above 10 kPa. Perfusion was switched to recirculation mode as soon as the blood had been flushed from the perfused liver (approx. 20 s). Zero time was taken as that of cannulation. The isolated liver *in situ* was covered with plastic film to prevent desiccation and cooling by evaporation, and allowed to stabilize for 3 min while the preparation was placed in the spectrometer cradle (heated with a water jacket) and an NMR spectroscopy single-turn radio-frequency coil secured in place on the surface of the right lobe. The preparation was then allowed to stabilize for a further 20 min, during which period the assembly was inserted in to the NMR spectrometer (see below) and tuning of the coil and shimming of the magnet were performed.

The erythrocyte-free perfusate at $36 \pm 1^\circ\text{C}$ (the temperature gradient across the liver was less than 1°C) consisted of Krebs bicarbonate buffer [11] gassed with O_2/CO_2 (19:1) to give pH 7.4. The substrate mixture consisted of sodium L-(+)-lactate (1.3 mM) and sodium palmitate (0.8 mM), bound to BSA (20 g/l). When a perfusion pH of 6.8 was required, this was achieved by replacement of the appropriate amount of NaHCO_3 with NaCl. Perfusate samples (5 ml) were taken at 35, 40 and 45 min from the portal and hepatic venous perfusion lines, the perfusate being mixed quickly with 1 ml of aqueous 20% (v/v) HClO_4 and frozen at -70°C until assays were performed; samples were also obtained for pH, p_{CO_2} and p_{O_2} . Flow rate was measured at the same time as perfusate sampling.

In separate studies, livers from animals prepared under conditions identical with those in groups 1 and 3 were removed for the estimation of total glucokinase (EC 2.7.1.2) and hexokinase (EC 2.7.1.1) activities (see below).

Mapping of intralobular glucose and ketone body production and pH_i

We have previously described this procedure in detail for a non-recirculating perfusion system [8]. However, because of the cost of the albumin buffer used, the current studies were performed with a mainly recirculating perfusion system. Baseline portal and hepatic venous samples were obtained as above for the estimation of net glucose output by the Fick principle. At 50 min, volumes (1–60 ml) of digitonin (4 g/l in Tris/HCl buffer, pH 7.4), different for each liver, were perfused retrogradely at approx. 30 ml/min. Digitonin binds plasma membrane cholesterol avidly on a first-pass basis, permeabilizing cells along the digitonin front, which moves retrogradely along the sinusoid more slowly than the

medium. The affected cells lose most of their cytosolic components, including enzymes not bound to organelles [12]. Mitochondria become grossly swollen; metabolic functions such as gluconeogenesis cease in affected cells. Cells that have not been reached by the digitonin front are normal histologically, electron microscopically [12,13] and functionally in terms of gluconeogenesis from lactate [8]. The demarcation of digitonin-permeabilized from digitonin-unaffected cells is sharp [12,13]. Flow was then resumed in the normal anterograde direction in a non-recirculating manner at approx. 80–90% of the flow rate before perfusion with digitonin, until portal pressure and hepatic swelling had subsided (approx. 5 min), when full anterograde flow was re-established. Immediately after the restoration of anterograde flow, 300 ml of the buffer in the perfusion system was replaced with fresh buffer (the total volume of the system was 700 ml). This ensured that the difference in concentration in substrates between the periods before and after digitonin sampling was less than 5%. There was no significant difference between groups in the concentration of lactate and glucose (accumulated from gluconeogenesis) in the perfusate (results not shown). It can be calculated from published results that the same is likely to be true for palmitate [14]. At 60 min (10 min after digitonin) a recirculating perfusion system was re-established. A number of control studies were performed in livers from group 3 in which the same protocol was followed, except that digitonin-free buffer was perfused retrogradely for 2 min before the restoration of anterograde flow, to establish the stability of the perfused liver under the same conditions but in the absence of digitonin. Portal and hepatic vein sample pairs were then taken at 60, 65 and 70 min for the estimation of net glucose and ketone body production in the post-digitonin liver. The liver was then fixed by perfusion at approx. 10 ml/min with 10% formalin and blocks were removed for the preparation of sections stained with haematoxylin and eosin. The proportion of destruction by area was then determined in each liver by semi-automated histomorphometry [8].

NMR spectroscopy

The perfusions were performed in a Sisco 4.7T NMR spectrometer so that the mapping of pH_i could be achieved with ^{31}P -NMR. The magnet had a horizontal bore 220 mm in diameter. A single-turn surface coil 11 mm in radius was placed directly on the liver surface and tuned to 81 MHz. Spectra were collected with the use of hard 90° pulses, arranged to maximize the liver signal, of 70 μs duration with a 6 kHz sweep width and an acquisition time of 0.33 s with no relaxation delay, producing a recycling time of 0.33 s, thus saturating the P_i signal from extracellular space, which has a considerably longer longitudinal relaxation time (T_1). Spectra were processed with 5 Hz exponential line broadening and referenced to the α -ATP plus α -ADP (-7.57 p.p.m.) peak and to the methylene diphosphonic acid (approx. 20.95 p.p.m.) external standard, as described previously [8]. Chemical shifts were measured by a 'nearest line' algorithm, which identifies the highest point on the P_i peak; pH_i was thus derived from the chemical shift of the P_i peak. We have previously demonstrated [8] that effectively all NMR-visible P_i is lost from cells affected by digitonin; thus the corresponding signals from livers after perfusion with digitonin did not contain appreciable contributions from the affected zones.

After initial shimming and collection of baseline spectra, the liver was removed from the spectrometer for perfusion with digitonin or control buffer, as described above. The preparation was then replaced in the spectrometer and re-shimmed within 5 min. The accumulation time for a single spectrum was 10 min;

observations were made immediately before perfusion with digitonin and over the interval 15–25 min later.

Analytical procedures

For glucose, perfusate samples were thawed and neutralized to pH 7.4; glucose concentration was measured with the glucose oxidase/peroxidase antiperoxidase method [15]. The following methods were used for other metabolites: 3-hydroxybutyrate, a colorimetric modification (Boehringer Mannheim) of the method of Williamson et al. [16]; acetoacetate, the method of Williamson et al. [16]; blood and perfusate pH, p_{CO_2} and p_{O_2} , Instrumentation Laboratories 1304 blood gas analyser. All measurements on samples stored at -70°C were made within 4 weeks, with the exception of acetoacetate, for which a storage limit of 2 weeks was set after preliminary studies showing that a mean of 84% recovery was achieved after 4 weeks. Glucokinase and hexokinase activities were measured as described previously [9].

Calculations and statistical methods

Net hepatic output or uptake of metabolites was calculated by the Fick principle. For oxygen consumption a solubility coefficient of 0.024 ml/ml was used to obtain the perfusate oxygen content from partial pressures. Results are expressed as means $\pm$ S.E.M. Two-tailed paired or unpaired *t* tests were used as appropriate. Net metabolite outputs before and after perfusion with digitonin were calculated as the means of three estimations before digitonin and three measurements after digitonin respectively. For technical reasons, not all studies produced complete data sets; all measurements made were nevertheless included in the analyses.

Primary plots were then made of the fraction remaining after perfusion with digitonin, of the net metabolite output or concentration (after digitonin compared with before digitonin) against the mean fractional volume of the lobule (and therefore the fractional volume of liver) remaining unaffected by digitonin [fractional volume remaining (FVR)]. FVR was determined by raising the fractional area destroyed as measured by histomorphometry to the power 3/2 and subtracting this from unity. This assumed that lobules were spherical, with the central hepatic venule at the centre.

RESULTS

In group 1 ($n = 20$) and group 2 ($n = 19$) animals, whose treatment differed only in the perfusate pH of the subsequent perfusion, no significant differences were found between the

groups, as expected. In blood taken from the inferior vena cava before perfusion, the mean $\pm$ S.E.M. venous blood pH values were 7.31 ± 0.01 and 7.34 ± 0.01 in groups 1 and 2 respectively; the corresponding values of mean p_{CO_2} were 7.11 ± 0.06 and 7.32 ± 0.05 kPa; mean $\pm$ S.E.M. venous blood glucose values were 4.87 ± 0.29 and 5.00 ± 0.42 mM in groups 1 and 2 respectively. Mean blood 3-hydroxybutyrate and acetoacetate concentrations were 1.49 ± 0.14 and 0.37 ± 0.03 mM respectively in group 1, and 1.36 ± 0.14 and 0.35 ± 0.11 mM respectively in group 2. Group 3 ($n = 16$) showed severe hyperglycaemia, acidosis and ketosis [mean blood glucose 25.1 ± 2.1 mM; venous blood pH 6.82 ± 0.04 ; p_{CO_2} 5.55 ± 0.2 kPa; 3-hydroxybutyrate 5.46 ± 0.43 mM; acetoacetate 0.67 ± 0.024 mM ($n = 12$)].

Pre-digitonin observations in the perfused liver

Results are shown in Table 1. The pH_i values (means $\pm$ S.E.M.) were 7.29 ± 0.01 , 6.91 ± 0.02 and 7.05 ± 0.03 in group 1 (normal, perfused at pH 7.4), group 2 (normal, perfused at pH 6.8) and group 3 (DKA, perfused at pH 6.8) respectively. Mean pH_i values in all groups were highly significantly different from each other ($P < 0.001$). Mean hepatic glucose output was identical in groups 1 and 3, and somewhat, but insignificantly ($P = 0.07$ and 0.09 respectively), smaller in group 2, the trend being consistent with the protection of gluconeogenesis in DKA against acidotic inhibition seen previously [7]. Mean 3-hydroxybutyrate output was very similar in the three groups but acetoacetate output was significantly greater ($P < 0.01$) in group 3 than in group 2. Mean oxygen consumption was significantly greater in group 3 than in groups 1 and 2 ($P = 0.03$ and $P = 0.02$ respectively). Mean hepatic venous p_{O_2} in the intact perfused liver was 11.4 ± 0.7 kPa ($n = 38$).

Mean glucokinase activity in whole livers was 1.80 ± 0.10 and 0.69 ± 0.26 unit/g liver in animals starved for 48 h ($n = 3$) and animals with DKA ($n = 6$) respectively ($P < 0.01$). Mean hexokinase activity was similar in DKA and control animals: 0.40 ± 0.13 and 0.36 ± 0.15 unit/g respectively in the same samples.

Mapping function and pH_i within the hepatic lobule

Control experiments in which digitonin-free buffer was perfused for 2 min retrogradely in a protocol otherwise identical with that of the mapping procedure showed no significant change in any variable. In three such studies, means $\pm$ S.E.M. for the ratio after and before perfusion were as follows: glucose output 0.98 ± 0.11 ; 3-hydroxybutyrate output 1.02 ± 0.08 ; acetoacetate output 0.95 ± 0.13 ; oxygen uptake 1.00 ± 0.04 . Mean ΔpH_i was 0.00 ± 0.03 . These results show that the fractional function is

Table 1 Metabolic data from perfused liver before perfusion with digitonin

Results are means $\pm$ S.E.M. Significances: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (comparisons with group 1); † $P < 0.05$, †† $P < 0.001$ (group 2 compared with group 3). Values in parentheses refer to the number of livers studied.

	Group		
	1	2	3
Source of rats	Normal	Normal	DKA
Perfusate pH	7.4	6.8	6.8
Glucose output ($\mu\text{mol}/\text{min}$ per 100 g body weight)	0.97 ± 0.09 (17)	0.74 ± 0.08 (18)	0.97 ± 0.10 (16)
3-Hydroxybutyrate output ($\mu\text{mol}/\text{min}$ per 100 g body weight)	2.80 ± 0.21 (20)	3.04 ± 0.18 (18)	2.69 ± 0.23 (15)
Acetoacetate output ($\mu\text{mol}/\text{min}$ per 100 g body weight)	0.63 ± 0.08 (12)	0.53 ± 0.05 (14)	† 0.77 ± 0.07 (14)
Oxygen consumption ($\mu\text{mol}/\text{min}$ per 100 g body weight)	17.94 ± 1.79 (14)	18.37 ± 1.15 (16)	$20.27^* \pm 1.30$ (14)
Mean pH _i	7.29 ± 0.01 (19)	6.91 ± 0.02 (14)***	$7.05 \pm \dagger\dagger\dagger 0.03$ (12)***

Figure 1 Effect of perivenous digitonin destruction on hepatic glucose output in starved and diabetic ketoacidotic rats

Upper panel: fraction of glucose output remaining (FGO) after retrograde perfusions with digitonin of various durations, plotted against average FVR. Each point is derived from a single liver (●, livers from normal rats perfused at pH 7.4; ○, livers from DKA rats perfused at pH 6.8). Points above the horizontal line denote that net glucose output was increased after digitonin. The solid curves through the group 1 (Gp 1) and group 3 (Gp 3) data in the upper panel represent respectively the fitted equations $FGO = 1 + 3.63(1 - FVR) - 4.86(1 - FVR)^{3/2}$ and $FGO = 1 - 0.87(1 - FVR)^{2/3}$. Lower panel: equivalent data from group 2 livers (from normal animals, but perfused at pH 6.8). The solid curve represents the fitted equation $FGO = 1 - 0.91(1 - FVR)^2$. See the Appendix for a description and explanation of the curve-fitting. Both curves pass through the (1,1) point (■) (see Appendix).

unity at FVR = 1.0. These results also validate the curve-fitting constraint through the (1,1) point (see the Appendix).

Glucose output

Figure 1 (upper panel) shows the results arising from the digitonin-based mapping procedure applied to glucose output in group 1 and 3 studies. Each point is a result from a different liver and refers to the fractional change in glucose output when FVR is as indicated on the abscissa. Points at the extreme right represent studies with the smallest degrees of destruction; those at the extreme left are from observations at the highest degrees of destruction, in which only a thin shell of periportal cells remained. For example, in Figure 1 the filled circles are the data from group 1 (controls perfused at pH 7.4); the data at FVR = 0.8 are livers from which 20% of the perivenous volume of the

Figure 2 Effect of lobular radius on gluconeogenic function per unit volume of cells relative to the periportal maximum, calculated as explained in the Appendix

For each curve the value at the most periportal location studied (the minimum FVR) was taken as unity; these minimum FVRs were as follows: normal livers [perfused at pH 7.4, group 1 (Gp 1)], 0.14; normal livers [at pH 6.8, group 2 (Gp 2)], 0.18; DKA livers [at pH 6.8, group 3 (Gp 3)], 0.11. The 95% confidence limits are shown for each curve and are calculated as described in Appendix 2 in [8].

liver had been 'removed', such that metabolite measurements represent the fractional function (compared with that before perfusion with digitonin) of the whole of the 80% of the liver volume periportal to that limit. It can be seen that in group 1 such small destructions produced a substantial increase in glucose output (by up to 40% in some livers) and it was not until FVR was less than 0.5 that glucose output clearly declined. That rapid decline indicates that glucose was produced periportal. Because hepatic glucose output increased when 20% of the liver was removed, the results indicate that glucose formed in periportal gluconeogenesis passed down the sinusoid and that a fraction was taken up in the more distal perivenous cells. Thus net hepatic glucose output was less than that synthesized by gluconeogenesis in group 1. This observation is similar to that previously shown by us in normal livers in which the sole substrate was 6 mM lactate [9]. In contrast, in group 3 (Figure 1, upper panel), glucose output declined at even small degrees of destruction, and somewhat more steeply than at larger degrees of destruction. It can be concluded that in DKA livers the perivenous cells, in addition to the periportal cells, contribute substantially to glucose output. Group 2 (Figure 1, lower panel) produced an intermediate result, with little if any perivenous glucose uptake and with points lying between the results for groups 1 and 3.

These conclusions can be demonstrated graphically by fitting curves to the results in Figure 1. These curves can then be used for the generation of point-by-point maps of function per unit volume of cells along the radius of the lobule. This procedure is outlined in the Appendix, and in more detail in Appendixes 1 and 2 in [8]. These point-by-point 'maps' are a graphical representation (Figure 2) of the rate of change of function with radius at each point along the radius. The curve for group 1 shows that glucose output declined from its maximum in the extreme periportal region until it switched over to glucose uptake at a point 65% along the radius from the lobular centre. In contrast, in group 3 (DKA), glucose output rises with increasing steepness in cells that were located progressively more perivenously.

Figure 3 Effect of perivenous destruction by digitonin on mean pH_i in the remaining viable part of the lobule

The results represent the change from mean pH_i in the liver before digitonin. Each point is derived from a single liver. Note that the symbols have different meanings from those in other figures, for reasons of clarity: ●, livers from normal rats perfused at pH 7.4 (group 1); ○, livers from normal rats perfused at pH 6.8; ▲, livers from DKA rats perfused at pH 6.8. The horizontal line through the point of zero change is that which would be obtained in the absence of zonal heterogeneity of pH_i .

pH_i

Figure 3 shows the data for the change in pH_i after perfusion with digitonin. In group 1, as destruction increased, there was little change in overall pH_i of the residual lobule until FVR = 0.6. At greater degrees of destruction there was a marked elevation of pH_i , similar to that observed in normal livers perfused with 6 mM lactate as the sole substrate [8]. In contrast, in group 3 the overall pH_i of the remnant decreased at small degrees of destruction and then remained unchanged at higher degrees of destruction. It can be inferred that the highest values of pH_i in DKA livers are found perivenously, in contrast with those in normal livers, in which the highest values are periportal. The values of pH_i observed at the highest degrees of destruction approximate to those existing in the most periportal zones, because they were not affected by more perivenous pH_i ; these values were in the range 7.4–7.7 for normal livers perfused at pH 7.4, and 6.55–6.9 for DKA livers perfused at pH 6.8. In group 2 there was little change in pH_i over the whole range of destruction.

Ketone bodies

Figure 4 (upper panel) shows the mapping data of 3-hydroxybutyrate production in the three groups. No significant differences are seen between the groups. The best-fit curves are straight lines (results not shown) and all lie entirely below the horizontal line through the (1,1) point, indicating the approximately uniform production of 3-hydroxybutyrate throughout the lobule. A very different picture is seen in Figure 4 (lower panel), which shows the plots of the acetoacetate mapping in the three groups. Moderate destructions produced a substantial increase in acetoacetate production, with larger destructions producing a decrease. It can be shown that, when FVR > 0.7, the mean fraction of acetoacetate output remaining was significantly greater than unity ($P = 0.023$), in contrast with the mean fractional 3-hydroxybutyrate output, which was significantly less than unity ($P = 0.003$). These findings (see the Discussion section) suggest

Figure 4 Effect of perivenous digitonin destruction on 3-hydroxybutyrate and acetoacetate production from starved and diabetic ketoacidotic rats

Upper panel: fraction of 3-hydroxybutyrate output remaining after retrograde perfusions with digitonin of various durations, plotted against average FVR. Each point is derived from a single liver. Lower panel: simultaneous observations of the fraction of acetoacetate output remaining. Symbols: ●, livers from normal rats perfused at pH 7.4; ▲, livers from normal rats perfused at pH 6.8; ○, livers from DKA rats perfused at pH 6.8; ■, the (1,1) point (see the Appendix).

that acetoacetate produced periportal is partly taken up perivenously, presumably for conversion to 3-hydroxybutyrate. There were no significant differences in the distribution of the points between the groups.

Oxygen consumption

Oxygen consumption is shown in Figure 5 (upper panel). In the normal livers perfused at both pH 7.4 and pH 6.8, only small changes in oxygen uptake occurred until destruction exceeded 30%, i.e. until FVR decreased to 0.7. The fitted curves for groups 1 and 2 are almost identical ($P = 0.73$). Oxygen uptake was therefore substantially lower in the perivenous zone than periportal despite adequate oxygen supply (venous pO_2 above 10 kPa; see the Materials and methods section). The group 3 curve is somewhat below that of the other groups, possibly indicating a trend toward greater perivenous oxygen uptake in DKA, but the evidence is weak ($P = 0.092$). In Figure 5 (lower panel) the method described in the Appendix has been used to derive point-by-point plots of oxygen consumption along the lobular radius. Because there is no clear difference between the

Figure 5 Fraction of oxygen consumption (FO_2) remaining after digitonin, plotted against FVR (top) and effect of lobular radius on oxygen consumption per unit volume of cells plotted as a fraction of that in the extreme periportal zone (bottom)

Top: the same symbols are used as in Figure 4; ■, the (1,1) point (see the Appendix). The fitted curves to the data are: group 1 (Gp 1), $FO_2 = 1 - 0.84(1 - FVR)^{3/2}$; group 2 (Gp 2), $FO_2 = 1 - 0.80(1 - FVR)^{3/2}$; group 3 (Gp 3), $FO_2 = 1 - 1.061(1 - FVR)^{3/2}$. Bottom: the FVR corresponding to the most periportal location studied was 0.11 (for explanation see the Appendix). For this Figure, all results in the upper panel were pooled and fitted by the curve $FO_2 = 1 - 0.87(1 - FVR)^{3/2}$ before transformation. The 95% confidence limits are shown, calculated as described in Appendix 2 in [8].

groups, all data have been pooled for this purpose. It can be seen that oxygen uptake per unit volume of cells at a point 30% along the radius from the lobular centre was only 20% of that in the extreme periportal region. As indicated above, absolute oxygen uptake before perfusion with digitonin (i.e. in intact perfused liver) was significantly greater in group 3 than in the other groups (Table 1).

DISCUSSION

The work described was undertaken to examine hepatic intra-lobular metabolism in DKA. The livers were obtained from animals in the starved state, which were likely to have been insulinopenic but with high levels of circulating glucagon and other catabolic hormones. Presumably these hormonal changes were particularly marked in the animals with DKA. No attempt was made to reproduce the hormonal environment in the perfusions.

The inclusion of a physiological concentration of non-esterified

fatty acid (palmitate) allowed us to address the question of whether the protection of hepatic pH_i in DKA still occurs in the presence of (partial) fatty acid oxidation and ketogenesis. Although hepatic pH_i measured during DKA in this study (before perfusion with digitonin) was lower than when measured in the absence of a ketogenic substrate [7], pH_i was still significantly protected compared with controls. In spite of a 4-fold lower concentration of lactate (1.3 mM compared with 5 mM lactate used in [7]), a trend towards increased hepatic glucose output in the DKA group compared with controls perfused at a similarly low pH remained. Notwithstanding the inclusion of palmitate, the control group perfused at pH 7.4 (group 1) exhibited the periportal production and perivenous uptake of glucose and the periportal elevation of pH_i observed previously [8,9].

One striking feature of the present observations is the ability of cells, at different sites within the liver lobule, to change their function in differing circumstances. Plasticity has previously been observed for a number of pathways, for example gluconeogenesis [17], fatty acid oxidation [18] and cytochrome P450 distribution [19] in isolated periportal and perivenous hepatocytes obtained from cold-exposed animals and under different nutritional states. However, the virtual reversal of aspects of cellular metabolism within hepatic lobular zones has not been described previously. In the present studies, with the use of the mapping technique in the intact 'remnant liver', the distributions of glucose metabolism and pH_i differed markedly between the groups. Thus in DKA, perivenous glucose uptake has been replaced by output, and gluconeogenesis predominates perivenously, in contrast with the situation in non-diabetic starved animals. The distribution of glucose output and uptake along the lobular radius in group 2 livers (from normal animals, perfused at pH 6.8) is intermediate between groups 1 and 3, indicating that only part of the difference between normal and DKA perfusions can be reproduced by acutely lowering the pH of the extracellular environment at the time of establishing the perfusion. It is of interest that Ikezawa et al. [20] found in isolated 'periportal' and 'perivenous' hepatocytes that the gluconogenic activity of the latter increased to that of the former when the hepatocytes were obtained from a rat model of type 1 diabetes.

The substantial shift of gluconeogenesis from the periportal to the perivenous region might be related to the gross reversal of the distribution of pH_i . In DKA the increased partial oxidation of fatty acids to acetoacetate should tend to decrease the periportal pH_i and thus inhibit gluconeogenesis in that region [2–6]. This suggestion is borne out by the marked decrease in pH_i observed in this study in the most periportal cells in livers from animals with DKA. In DKA livers, pH_i is more alkaline perivenously, perhaps partly because of the cessation of anaerobic glycolysis in this zone; the resulting increase in perivenous pH_i might permit gluconeogenesis from lactate to proceed. Once perivenous gluconeogenesis from lactate is established, further protons are consumed [10,21], and thus the high pH_i might become self-sustaining. [We have shown (S. P. Burns, H. C. Murphy, R. A. Iles and R. D. Cohen, unpublished work) that in livers with 50% perivenous destruction the high pH_i of the periportal zone is markedly lowered by removal of lactate from the perfusate and can be subsequently restored by its re-introduction.] For this explanation to be valid, these proton-consuming reactions would have to quantitatively exceed proton generation in perivenous ketogenesis; it can be calculated from the results in Table 1 and the fitted curves of Appendix 1 that this is indeed so.

The reason for the reversal of glucose handling in DKA might also be related to the loss of expression of glucokinase in this zone. The concentration of glucose reaching the perivenous cells in these studies did not exceed 0.15 mM, which is comparable

with the K_m for glucose of hexokinases I–III and lower than that of glucokinase (hexokinase IV, $K_m \approx 10$ mM). Enzyme activities were not mapped in the present study, although previous work [9] has shown that, in normal rats starved for 48 h, glucokinase is heavily concentrated in the most perivenous few cells, whereas hexokinase is evenly distributed throughout the lobule. Because total hexokinase (I–III) activity is not significantly altered, it seems unlikely that changes in hexokinase activity are involved. The marked decrease in total glucokinase activity in the DKA livers to approx. 35% of normal is compatible with the known half-life of glucokinase in cultured hepatocytes [22], assuming the rapid cessation of synthesis owing to insulinopenia after treatment with streptozotocin. It is uncertain whether or not this is relevant to the shift of gluconeogenesis to the perivenous zone in DKA.

It has previously been shown with surface measurements that p_{O_2} is low in the perivenous region [23]. We have now produced a detailed description of the variation in oxygen consumption along the radius of the hepatic lobule, albeit under particular conditions of the present studies. An important finding is that oxygen consumption is very low in the most perivenous region (particularly in the livers from normal animals), despite the artificially high perivenous p_{O_2} in these liver perfusion studies. This suggests that the perivenous cells are normally relatively poorly equipped for oxidative metabolism and thus might rely heavily on glycolysis for ATP production. This conclusion could be related to the observation that oxygen tension at physiological levels is a powerful negative modulator of insulin-stimulated glucokinase gene transcription [24,25]. Thus the low perivenous p_{O_2} could ensure relatively high levels of glucokinase to permit the glycolytic synthesis of ATP in this zone. It should be noted that under the present conditions there is no stimulus to oxygen uptake through the cytochrome P450 mono-oxygenase system, which is located predominantly perivenously [19].

The marked increase in acetoacetate production seen after small perivenous destructions suggests that acetoacetate produced periportal is partly converted perivenously to 3-hydroxybutyrate. To be certain about this it is necessary to demonstrate that digitonin-affected cells cannot perform this conversion because 3-hydroxybutyrate dehydrogenase is a mitochondrial enzyme, some of which are known to be partly retained after treatment with digitonin [12]. In two studies (results not shown) we have demonstrated that complete destruction of the hepatic lobules by digitonin decreases ketogenesis from palmitate to non-measurable levels (less than 10% of the level before digitonin). With ketone bodies provided as substrates, the addition of 3 mM NADH to the perfusate produces a non-significant trend to the generation of small amounts of 3-hydroxybutyrate, suggesting that, although some 3-hydroxybutyrate dehydrogenase might remain active in digitonin-destroyed cells, the reduction of acetoacetate is limited in these cells by the loss of NADH.

We conclude that the perivenous conversion of periportal generated acetoacetate to 3-hydroxybutyrate is likely to be a physiological phenomenon, the predominantly perivenous distribution (histochemically determined) of 3-hydroxybutyrate dehydrogenase [26,27] presumably accounting for this phenomenon. However, it can be estimated that a major fraction of the 3-hydroxybutyrate formed in the perivenous 30% of lobular volume must be generated perivenously from fatty acid oxidation *de novo*. Some of the NAD^+ required might be derived from the reduction of acetoacetate to 3-hydroxybutyrate, the acetoacetate being derived both periportal and from local β -oxidation. The mitochondrial redox potential is therefore likely to increase across the perivenous zone, which is consistent with the relatively low oxygen uptake in this zone.

It may be noted that other zonation changes observed both in the present study and elsewhere seem to provide the pH and enzymic environment required for the perivenous shift of gluconeogenesis seen in DKA livers. Perivenous fatty acid oxidation presumably supplies the acetyl-CoA required [28] as an obligatory activator for pyruvate carboxylase, an enzyme with a high flux control coefficient in gluconeogenesis from three-carbon intermediates. Furthermore, the activation of pyruvate carboxylase by acetyl-CoA is stimulated by raising pH in the physiological range [28]. Although this is an observation *in vitro*, the finding that the effect of changes in pH on hepatic gluconeogenesis from lactate is exerted during the conversion of pyruvate to oxaloacetate [6] is consistent with its being of physiological importance. Information on the intralobular distribution of pyruvate carboxylase itself and on fructose 1,6-bisphosphatase, also essential for gluconeogenesis, is limited to one study each [29], in isolated hepatocytes from animals starved for 24 h, showing in both cases a modest predominance in periportal cells. Another enzyme specific to gluconeogenesis, phosphoenolpyruvate carboxykinase, is predominantly located periportal in the fed state but has been demonstrated both by enzyme histochemistry [30] and the present mapping technique [9] to be more generally distributed in the starved state.

Our earlier suggestion [7], that the unique protection of pH_i in DKA was the reason that gluconeogenesis was able to proceed so vigorously in DKA in spite of the increasingly severe systemic acidosis, can now be developed to take into account the zonation of metabolism and pH_i described here. Thus we have now shown that in DKA many of the essential conditions for gluconeogenesis from lactate are present in the perivenous zone; this might account for the observation that in this condition gluconeogenesis occurs throughout the lobule, in contrast with the normally exclusive periportal location.

We thank Dr. Paul Kinchesh (University of London Intercollegiate Research Service, Queen Mary and Westfield College, London, U.K.) for the provision of NMR facilities. This work was supported by a project grant from the Wellcome Trust to RDC, SPB and RAI.

REFERENCES

- Forbath, N. and Hetenyi, G. (1966) *Diabetes* **15**, 778–789
- Hems, R., Ross, B. D., Berry, M. N. and Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
- Lloyd, M. H., Iles, R. A., Strunin, J., Layton, J., Simpson, B. R. and Cohen, R. D. (1973) *Clin. Sci.* **45**, 543–549
- Iles, R. A., Cohen, R. D. and Baron, P. G. (1978) *Clin. Sci.* **55**, 175–181
- Kashiwagura, T., Deutsch, C. J., Taylor, J., Erecinska, M. and Wilson, D. F. (1984) *J. Biol. Chem.* **259**, 237–243
- Iles, R. A., Cohen, R. D., Rist, A. H. and Baron, P. G. (1977) *Biochem. J.* **164**, 185–191
- Beech, J. S., Williams, S. R., Cohen, R. D. and Iles, R. A. (1989) *Biochem. J.* **263**, 737–744
- Burns, S. P., Cohen, R. D., Iles, R. A., Germain, J. P., Going, T. C. H., Evans, S. J. W. and Royston, P. (1996) *Biochem. J.* **319**, 377–383
- Burns, S. P., Desai, M., Cohen, R. D., Hales, C. N., Iles, R. A., Germain, J. P., Going, T. C. H. and Bailey, R. A. (1997) *J. Clin. Invest.* **100**, 1768–1774
- Cohen, R. D., Iles, R. A., Barnett, D., Howell, M. E. O. and Strunin, J. (1971) *Clin. Sci.* **41**, 159–170
- Krebs, H. A. and Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–36
- Racine, L., Scoazec, J., Moreau, A., Bernuau, D. and Feldmann, G. (1993) *J. Histochem. Cytochem.* **7**, 991–1001
- Quistorff, B. and Romert, P. (1985) *Histochemistry* **92**, 487–491
- Clark, C. M. and Scow, R. O. (1970) *Diabetes* **19**, 924–929
- Trinder, P. (1969) *J. Clin. Pathol.* **22**, 158–161
- Williamson, D. H., Mellanby, J. and Krebs, H. A. (1962) *Biochem. J.* **82**, 90–98
- Shiota, M., Fujimoto, Y., Inagama, M., Hiramatsu, M., Moriyama, M., Kimura, K., Ohta, M. and Sugano, T. (1993) *Am. J. Physiol.* **265**, E559–E564
- Guzman, M., Bijleveld, C. and Geelen, M. J. H. (1995) *Biochem. J.* **311**, 853–860
- Oinonen, T. and Lindros, K. O. (1998) *Biochem. J.* **329**, 17–35

- 20 Ikezawa, Y., Yamatani, K., Ogawa, A., Ohnuma, H., Igarashi, M., Daimon, M., Manaka, H. and Sasaki, H. (1998) *J. Lab. Clin. Med.* **132**, 547–555
- 21 Cohen, R. D. (1990) in *The Metabolic and Molecular Basis of Acquired Disease* (Cohen, R. D., Lewis, B., Alberti, K. G. M. M. and Denman, A. M., eds.), pp. 962–1001, Baillière Tindall, London
- 22 Ilyedjian, P. B., Jotterand, D., Nouspikel, T., Asfari, M. and Pilot, P. (1989) *J. Biol. Chem.* **264**, 21824–21829
- 23 Matsumura, T. and Thurman, R. G. (1983) *Am. J. Physiol.* **244**, G656–G659
- 24 Kietzmann, T., Roth, U., Freimann, S. and Jungermann, K. (1997) *Biochem. J.* **321**, 17–20
- 25 Krones, A., Kietzmann, T. and Jungermann, K. (1998) *FEBS Lett.* **421**, 136–140
- 26 Teutsch, H. F., Altemus, J., Gerlach-Arbeiter, S. and Kyander-Teutsch, T. L. (1992) *J. Histochem. Cytochem.* **40**, 213–219
- 27 Novikoff, A. B. (1959) *J. Histochem. Cytochem.* **7**, 240–244
- 28 Scrutton, M. C. and Utter, M. F. (1967) *J. Biol. Chem.* **242**, 1723–1735
- 29 Jones, C. G. and Titheradge, M. A. (1996) *Arch. Biochem. Biophys.* **326**, 202–206
- 30 Jungermann, K. (1986) *Acta Histochem. Suppl.* **32**, 89–98
- 31 Press, W. H., Flannery, B. P., Teukolsky, S. A. and Vetterling, W. T. (1988) *The Art of Scientific Computing: Numerical Recipes in C*, pp. 542–547, Cambridge University Press, New York

APPENDIX

Each point in the graphs of data (e.g. Figure 1) represents the function of the whole of the intact portion of the lobule after digitonin. To give estimates of metabolic activity and metabolite concentration at each point along the radius of the hepatic lobule, these plots can be transformed as described in [8]. This is a simple procedure that merely calculates the rate of change of activity or function at each successive value of FVR by differentiation of the curves fitted to the data. These transformations have been computed here for the glucose and oxygen data. For reasons already given [8], it is not possible to perform these transformations for cell pH, although some inferences concerning intralobular pH distribution can be made from the pH_i data.

Curves were first fitted to the plots of fractional metabolite output remaining against FVR by using the Levenburg–Marquandt algorithm [31]. The curves were chosen to pass through the (1,1) point on this plot, which indicates that when the lobule is intact ($\text{FVR} = 1.0$), the fractional function is 1.0 (i.e. function is unchanged). This constraint has already been justified for several variables under control conditions when the substrate was sodium lactate (6 and 1.5 mM), including glucose output, P_i and pH_i [8] and again for the same variables, together with 3-hydroxybutyrate, acetoacetate and oxygen uptake, in the present

study. When the same number of curve-fitting parameters were used for all three groups, the curves were chosen by minimum sum of squares; if different numbers of parameters were used for different groups, an F test was used to determine whether an extra parameter improved the fit significantly. The method used to determine whether the data in different groups required different models to be fitted has been described previously [9]. For glucose, the P value for each of the three groups requiring a different model was less than 0.001. For oxygen uptake, there was no evidence that different models were necessary, though there was a trend towards the data for group 3 being located below that of groups 1 and 2 ($P = 0.092$). It is important to note that the equations of the fitted curves imply no specific physiological model; they are merely vehicles for the transformation procedure to allow the data to be represented as ‘activity’ at each point along the radius of the lobule rather than as ‘activity’ in the remnant liver that is described by the data. Overall rates of metabolic pathways along the radius of the liver lobule in the transformed data (Figures 2 and 5, lower panel) are expressed relative to that in the most proximal periportal region studied; the corresponding FVR at which this point was chosen is given in the figure legends.

Received 12 April 1999/1 July 1999; accepted 27 July 1999