Zonation of hepatic lipogenic enzymes identified by dual-digitonin-pulse perfusion

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The zonal distribution within rat liver of acetyl-CoA carboxylase, ATP citrate-lyase and fatty acid synthase, the principal enzymes of fatty acid synthesis, was investigated by using dual-digitonin-pulse perfusion. Analysis of enzyme mass by immunoblotting revealed that, in normally feeding male rats, the periportal/perivenous ratio of acetyl-CoA carboxylase mass was 1.9. The periportal/perivenous ratio of ATP citrate-lyase mass was 1.4, and fatty acid synthase exhibited the largest periportal/perivenous mass gradient, having a ratio of 3.1. This pattern of enzyme distribution was observed in male rats only; in females, the periportal/perivenous ratio of enzyme mass was nearly equal. The periportal/perivenous gradients for acetyl-CoA carboxylase, ATP citrate-lyase and fatty acid synthase observed in fed (and fasted) males were abolished when animals were fasted (48 h) and refed (30 h) with a high-carbohydrate/low-fat diet. As determined by enzyme assay of eluates obtained from the livers of normally feeding male rats, there is also periportal zonation of acetyl-CoA carboxylase activity, expressed either as units per mg of eluted protein or units per mg of acetyl-CoA carboxylase protein, suggesting the existence of gradients in both enzyme mass and specific activity. From these results, we conclude that the enzymes of fatty acid synthesis are zoned periportally in the liver of the normally feeding male rat.

INTRODUCTION

The liver parenchyma contains subpopulations of hepatocytes which differ with respect to a number of histological characteristics [1] and to the degree of expression of a number of enzymes [2]. This latter difference forms the basis of the hypothesis of metabolic zonation [2,3], according to which there is a functional difference in activity of several major metabolic pathways in the periportal and perivenous zones of the mammalian liver. Zonation has been most extensively studied for carbohydrate and nitrogen metabolism; there is much evidence suggesting that the processes of gluconeogenesis [4–7], urea synthesis [8–11], and glutamine hydrolysis [8,10] are primarily periportal, whereas other processes, e.g. glycolysis [12,13], glutamine synthesis [8,10,14], and the cytochrome *P*-450 system [15], are exclusively or predominantly perivenous. Data conflicting with this view have appeared concerning the zonation of gluconeogenesis [16], glycolysis [17,18] and urea synthesis [19]; for review, see [20].

Unlike carbohydrate and nitrogen metabolism, relatively little is known regarding the possible zonation of the key enzymes of fatty acid biosynthesis, acetyl-CoA carboxylase (ACC), ATP citrate-lyase (ACL) and fatty acid synthase (FAS). Katz and co-workers have reported that the activities of both ACC and ACL are higher in perivenous hepatocytes, as determined in microdissected rat liver tissue [21,22]. There is also evidence that the activities of the enzymes controlling metabolic pathways involved in the provision of reducing equivalents for fatty acid synthesis, such as glucose-6-phosphate de-

hydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme, are also higher in the perivenous zone [23,24]. In addition, in the liver of normally feeding rats, 3-hydroxy-3-methylglutaryl-CoA reductase, the principal regulatory enzyme of sterol biosynthesis, and 3-hydroxy-3-methylglutaryl-CoA synthase have been found to be principally confined to periportal hepatocytes [25,26]. It is possible that the zonation of key lipogenic enzymes may be important in understanding the regulation of lipogenesis and lipoprotein metabolism.

Many earlier investigations of liver heterogeneity have relied on histochemistry, immunohistochemistry, and/or microdissection of freeze-dried sections. These methods have a number of inherent drawbacks, including fixation artifacts, difficulty in quantification, and antibody specificity. In addition, microdissection is a tedious and time-consuming technique that relies on anatomical markers independent of hepatic microcirculation. In this investigation, we have elected to use the recently developed dual-digitonin-pulse perfusion method to overcome some of these limitations [27]. This method allows separate sampling of the cytosolic eluate from periportal or perivenous hepatocytes from the same liver and takes advantage of the physiological microcirculation to distinguish these hepatic zones. It is highly selective for either periportal or perivenous cytosol, as judged by the presence of marker enzymes [27,28] with zonal gradients established by microdissection such as glutamine synthase [14] and alanine aminotransferase [29]. Careful control of flow rate, temperature, digitonin concentration and duration of digitonin pulse minimizes contamination of the eluted fractions by mitochondrial components.

Abbreviations used: ACC, acetyl-CoA carboxylase; ACL, ATP citrate-lyase; FAS, fatty acid synthase; PAGE, polyacrylamide-gel electrophoresis; LDH, lactate dehydrogenase.

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Employing quantitative immunoblotting of periportal and perivenous eluates obtained by this method, we report here the periportal zonation of ACC, FAS and ACL in the male rat liver. In contrast, there is no apparent zonation discernible by this technique in the livers of female rats.

EXPERIMENTAL

Materials

Chemicals were purchased from Sigma (St. Louis, MO, U.S.A.), Fisher (Fair Lawn, NJ, U.S.A.) and Mallinckrodt (Paris, KY, U.S.A.). Electrophoresis equipment and reagents were purchased from Bio-Rad (Richmond, CA, U.S.A.), and nitrocellulose (BA83) was from Schleicher and Schull (Keene, NH, U.S.A.). ^{125}I -protein A and $\text{NaH}^{14}\text{CO}_3$ were purchased from ICN (Irvine, CA, U.S.A.), and ^{125}I -labelled goat anti-mouse Ig was from Du Pont NEN (Boston, MA, U.S.A.).

Dual-digitonin-pulse perfusion

Details of the perfusion system have been described previously [7,27]. Briefly, livers of Wistar rats were perfused *in situ* in the porta $\rightarrow$ cava direction with Krebs-Henseleit bicarbonate buffer (37 °C) equilibrated with O_2/CO_2 (19:1) for 10 min. After a 10 s pulse with digitonin (4–8 mg/ml), the direction of flow was reversed to cava $\rightarrow$ porta, and periportal samples were collected in four consecutive 10 s intervals (designated pp1–pp4). Perfusion flow continued in the cava $\rightarrow$ porta direction, and after a second pulse with digitonin the direction of flow was reversed to porta $\rightarrow$ cava to collect perivenous samples (designated pv1–pv4). The initial direction of perfusion was reversed for each experiment, so that similar numbers of experiments were performed with either antegrade (porta $\rightarrow$ cava) or retrograde (cava $\rightarrow$ porta) perfusion as the first step.

In initial experiments involving shipped samples, samples of the eluate fractions were minimally diluted in ice-cold sampling buffer consisting of (final concns.) 50 mM-potassium phosphate (pH 7.5), 2 mM-dithiothreitol, 2 mM-EDTA, 2 mM-EGTA, 50 mM-NaF, 2 mg of bovine serum albumin/ml, 4 μg of leupeptin/ml, 0.5 mM-phenylmethanesulphonyl fluoride and aprotinin (10 kallikrein-inactivator units/ml). Samples were then prepared for SDS/polyacrylamide-gel (SDS/PAGE) and immunoblotting [30]. Additional diluted samples were also frozen and freeze-dried for subsequent determination of enzyme activity. Protein content was measured in samples of eluate fractions, and was corrected for the bovine serum albumin content of the sampling buffer.

For determination of ACC activity in freshly prepared periportal and perivenous samples, eluate fractions were collected directly into a minimal volume of ice-cold buffer consisting of (final concns.) 50 mM-Tris/HCl (pH 7.5 at 4 °C), 2 mM-EDTA, 100 mM-NaF, 2 mM-dithiothreitol, 250 mM-sucrose and the following proteinase inhibitors (freshly added): antipain (4 μg /ml), aprotinin (10 kallikrein-inactivator units/ml), phenylmethanesulphonyl fluoride (0.5 mM), soya-bean trypsin inhibitor (4 μg /ml), tosyl-L-lysylchloromethane (3.5 μg /ml) and tosyl-L-phenylalanylchloromethane (3.5 μg /ml). For both periportal and perivenous samples, the two fractions containing the most protein were pooled and then centrifuged at 100 000 *g* for 60 min at 4 °C. The

Fig. 1. Characterization of antisera used for immunoblotting of ACC, FAS and ACL

Standard curves for each lipogenic enzyme were routinely prepared with increasing concentrations of purified enzyme. After SDS/PAGE on 7.2%-acrylamide gels and transfer to nitrocellulose, immunoblots were probed with sheep polyclonal antiserum directed against either ACC (a) or FAS and ACL (b). Immunoreactive material was detected by treatment with ^{125}I -Protein A followed by autoradiography as described in the Experimental section. ACC blot: 1, 20 ng; 3, 40 ng; 5, 60 ng; 7, 80 ng; 9, 160 ng; 11, 320 ng; 13, 640 ng; 15, 1.28 μg . FAS (odd-numbered lanes) and ACL (even-numbered lanes) blot: 1 and 2, 25 ng; 3 and 4, 50 ng; 5 and 6, 75 ng; 7 and 8, 100 ng; 9 and 10, 200 ng; 11 and 12, 300 ng; 13 and 14, 400 ng; 15, 500 ng.

supernatant was analysed by both ACC assay and immunoblotting (see below).

Electrophoresis and immunoblotting

SDS/PAGE (7.2%-polyacrylamide gels) and immunoblotting were performed essentially as described previously [30], except that Tween 20 (0.05%, v/v) was substituted for antifoam A in wash and blocking solutions. Preliminary experiments established that transfer conditions originally optimized for the transfer of ACC, a high-molecular-mass protein, were also suitable for the efficient transfer of ACL and FAS. After transfer and blocking, blots were incubated overnight with a 1:100 final dilution of the appropriate primary antibody (see below). After several washes to remove primary antibody that had not reacted, blots were incubated for 2 h with either 15 μCi of ^{125}I -Protein A (0.3 $\mu\text{Ci}/\text{ml}$; sp. radioactivity 5–10 $\mu\text{Ci}/\mu\text{g}$) when the polyclonal antiserum was the primary probe, or 5 μCi of ^{125}I -labelled goat anti-mouse Ig (0.1 $\mu\text{Ci}/\text{ml}$; sp. radioactivity 2–10 $\mu\text{Ci}/\mu\text{g}$) when the monoclonal antibody was the primary probe. Blots were washed four times to remove unbound protein A or second antibody and air-dried. Immunoreactive bands were made visible after

Fig. 2. Immunoblot analysis of elution pattern of ACC after dual-digitonin-pulse perfusion

The elution pattern of ACC after dual-digitonin-pulse perfusion was analysed by immunoblotting. Periportal (pp) and perivenous (pv) eluates from male rats fed *ad libitum* were collected in four consecutive 10 s intervals as described in the Experimental section. Samples for SDS/PAGE and immunoblotting were prepared by removing an equal volume (150 μ l) from each fraction and diluting (1:1) with 50 mM-Tris HCl (pH 7.5), and further diluting (5:1) with 0.24 M-dithiothreitol/6% (v/v) SDS/20% (w/v) sucrose. Sample lanes contained various amounts of eluate protein, ranging from 20 μ g (pp1) to 120 μ g (pp2). The autoradiogram presented here is the result of an individual experiment, and is representative of results obtained in four separate experiments. Eluates: pp1 and pv1, 0–10 s; pp2 and pv2, 10–20 s; pp3 and pv3, 20–30 s; pp4 and pv4, 30–40 s.

autoradiography for 15–24 h at -70°C by using Kodak XAR-5 preflashed film and a DuPont Cronex intensifying screen.

To quantify the amount of ^{125}I radioactivity in immunoreactive bands, the nitrocellulose was overlaid with the autoradiogram and the band of interest was excised and counted for radioactivity in a Beckman γ -radiation counter (model 4000). Background radioactivity was estimated by excising and counting radioactivity of regions of the nitrocellulose containing no visible immunoreactive bands. The linear range of this procedure was determined by preparing standard curves for increasing concentrations of purified ACC, ACL and FAS. The response was linear in the range of 25–1000 ng of ACC protein, 25–500 ng of FAS protein and 75–300 ng of ACL protein (Fig. 1). Care was taken to ensure that the amount of total eluate protein applied to gels yielded an amount of antigen within the linear range. Data are expressed either as c.p.m. of ^{125}I -Protein A associated with immunoreactive bands, or μ g of enzyme, as determined from the appropriate standard curve.

Antisera and enzyme preparation

Three primary antisera were utilized in this study: mouse monoclonal antibody and sheep polyclonal antiserum directed against rat liver ACC, and sheep polyclonal antiserum directed against rat liver FAS and ACL. The preparation of the anti-ACC monoclonal antibody (ACC.7) has been previously described [30]. Polyclonal antiserum against rat liver ACC was raised in sheep immunized with ACC isolated from livers of fasted/refed rats [31]. This antiserum does not react with rat liver FAS. ACL and FAS were probed with a single polyclonal antiserum that was raised in sheep immunized with a mixture of ACL and FAS purified from rat liver.

Fig. 3. Immunoblot analysis of elution pattern of FAS and ACL after dual-digitonin-pulse perfusion

Experimental details are as described in Fig. 2 legend.

This antiserum was kindly given by Dr. Joseph Avruch (Massachusetts General Hospital, Boston, MA, U.S.A.). ACC [32], ACL [33] and FAS [34] were isolated from livers of rats fasted for 48 h and refed for 48 h with a high-carbohydrate/fat-free chow (Bio-Serv, Frenchtown, NJ, U.S.A.).

Enzyme assays and protein determination

Enzymic assay of freeze-dried samples was performed as follows: samples of each eluate fraction diluted in sample buffer were freeze-dried and sent to Dartmouth. After reconstitution in 1 mM-dithiothreitol, samples were assayed for ACC, ACL and lactate dehydrogenase (LDH) activities. ACC activity was determined by measuring the amount of $\text{H}^{14}\text{CO}_3^-$ incorporated into acid-stable material [35], and ACL and LDH were assayed by spectrophotometric techniques [36]. FAS activity could not be measured owing to the presence of phenylmethanesulphonyl fluoride in the sampling buffer. ACC activity in the freshly isolated supernatant was assayed at 0, 0.5 mM- and 10 mM-citrate (V_{max}) [35]. Alanine aminotransferase and pyruvate kinase were assayed spectrophotometrically in fresh samples as described previously [28]. Protein was measured by the methods of Lowry *et al.* [37] or Bradford [38], with bovine serum albumin (Fraction V) as the protein standard.

RESULTS

The zonation of the lipogenic enzymes was initially examined in livers from male rats fed *ad libitum* with standard rat chow. The elution patterns in a typical experiment for ACC, ACL and FAS after dual-digitonin-pulse perfusion are shown in Figs. 2 and 3. Analysis by immunoblotting indicated that each enzyme was present in both periportal and perivenous eluates, although the concentration of each was greater in periportal samples. For both periportal and perivenous eluates, the peak elution of each enzyme occurred in the second 10 s collection interval (fractions pp2 and pv2). Although these data visually indicate an enhanced periportal concentration of each enzyme, they are not corrected for total protein in each eluate fraction. This correction (see below) is necessary in order to express the apparent gradient as a ratio of enzyme mass per weight of cytosolic protein. It has been previously shown that protein concentration (mg of total protein/ml of cytosol) is the same in periportal and perivenous cells [39], and it is

Fig. 4. Release of lipogenic-enzyme activity and mass

Periportal (PP) and perivenous (PV) eluate fractions were collected as described in the Experimental section. A 1.5 ml sample was taken from each fraction, freeze-dried, and assayed for ACC and ACL activity after reconstitution in 1 mM-dithiothreitol. An additional sample (150 μ l) was removed for SDS/PAGE and immunoblotting. The activities of ACC (assayed at 20 mM-citrate) and ACL are presented as munits per total volume of freeze-dried sample. Lipogenic-enzyme content in eluate fractions was determined by quantitative immunoblotting and is presented as c.p.m. ($\times 10^{-3}$) of 125 I-Protein A associated with ACC, ACL or FAS per total volume of the eluate fraction. Data points are averages of two separate experiments.

assumed that the elution of the enzymes under investigation is proportional to total protein elution. In four experiments performed in livers of normally feeding male rats, the total released protein from periportal perfusion was 38.0 ± 3.7 mg (mean $\pm$ S.E.M.), whereas 22.9 ± 2.0 mg was released from the perivenous perfusion.

Lipogenic-enzyme activity and mass co-eluted in both

Table 1. Lipogenic-enzyme content in periportal and perivenous eluates

The quantity of radioactive 125 I-Protein A associated with ACC, ACL and FAS in each eluate fraction was determined by quantitative immunoblotting (with polyclonal antisera) and is expressed as c.p.m./mg of cytosolic protein. Values for individual eluate fractions are averages of two separate experiments. The periportal:perivenous (PP:PV) ratio of the peak fractions represents the mean $\pm$ S.E.M. of (*n*) determinations performed with samples obtained in four separate experiments using male rats. Peak fractions as judged by protein content and enzyme activity (for ACC and ACL) refer to pp2 and pv2.

Eluate fraction ... Enzyme	$10^{-3} \times$ Enzyme content (c.p.m./mg of cytosolic protein)				PP:PV ratio (peak fractions)
	1	2	3	4	
ACC					
PP	5.4	10.9	16.7	17.6	
PV	4.1	5.7	6.0	8.8	
PP:PV	1.3	1.9	2.8	2.0	1.9 ± 0.1 (8)
ACL					
PP	0.9	1.4	1.2	1.1	
PV	0.2	0.9	0.7	0.5	
PP:PV	4.5	1.6	1.7	2.2	1.4 ± 0.2 (8)
FAS					
PP	8.4	12.7	12.3	7.3	
PV	2.1	2.9	2.7	2.1	
PP:PV	4.0	4.4	4.6	3.5	3.1 ± 0.5 (10)

periportal and perivenous fractions. The peak of both ACC and ACL activity was in fraction 2, and was greater in the periportal eluate (Fig. 4). In each case, the peak of enzyme activity corresponded to the peak of enzyme mass. FAS activity was not measurable in reconstituted samples. However, the peak of FAS mass as determined by immunoblotting showed a similar pattern of elution and distribution.

The mass of ACC, ACL and FAS relative to released protein in male rats is shown in Table 1. In two experiments, enzyme content was determined in all four individual eluate fractions; in subsequent experiments, only the peak fractions (as determined by released protein and enzyme activity) were analysed. These results demonstrate that the mass of each enzyme was consistently higher in periportal fractions, and that the periportal/perivenous ratio was relatively constant over the 10 to 40 s collection intervals. The estimate of periportal/perivenous gradient in the first 10 s interval (fraction 1) is subject to some variability, because of the very low content of each enzyme in these fractions. Analysis of the peak eluate fractions from four experiments revealed that the largest periportal/perivenous gradient of enzyme mass (corrected for protein content) was exhibited by FAS, which had a mean gradient of 3.1 ± 0.5 ($\pm$ S.E.M.; Table 1). The periportal/perivenous enzyme mass gradient for ACC was 1.9 ± 0.1 , and that for ACL 1.4 ± 0.2 . The gradients for all three enzymes were similar whether the initial perfusion was in the porta $\rightarrow$ cava direction or vice versa (results not shown; see below). In

Fig. 5. Distribution of ACC, ACL and FAS in peak eluate fractions from male and female rats

Peak periportal (PP) and perivenous (PV) eluate fractions obtained from male (M) and female (F) rats fed *ad libitum* were analysed by immunoblotting by using a monoclonal antibody against ACC (a) and a polyclonal antiserum against FAS and ACL (b). Samples were diluted to equalize protein concentrations (2.5 mg/ml) between both PP and PV samples and between males and females; 50 μ g of eluted protein was applied to sample lanes 1–4 and 6–9. Lanes 5, 10 and 11 respectively contain the following quantities of purified enzymes: ACC, 80 ng; FAS, 160 ng; ACL, 320 ng.

contrast with males, no zonation of the lipogenic enzymes was observed in female rats. Immunoblot analysis of peak eluate fractions indicated that the contents of ACC, ACL and FAS were equivalent in periportal and perivenous samples (Fig. 5).

Results from preliminary experiments suggested that, in addition to the periportal gradient in ACC mass, a second type of periportal gradient in actual specific activity existed. However, it is important to note that in these experiments ACC activity was measured in reconstituted freeze-dried samples. It is possible that alterations in enzyme activity could have occurred during freeze-drying, shipment, or reconstitution. Therefore, it was necessary to confirm these initial findings of a periportal gradient in the actual specific activity of ACC by measuring ACC activity in freshly prepared periportal and perivenous eluates. In agreement with the preliminary findings, there was a periportal/perivenous gradient of ACC activity (expressed as munits/mg of protein; Table 2). This gradient of activity was evident whether ACC was assayed in the absence or presence of 0.5 mM- and 10.0 mM-citrate, although the gradient was more pronounced at the lower citrate concentration. The periportal gradient persisted after correction for actual ACC mass as determined by immunoblotting, particularly in the absence of, or at low, citrate concentration. These data indicate that the total ACC gradient is accounted for by individual gradients in enzyme mass and actual specific activity. The magnitude and statistical significance of the activity gradient were unaffected by the initial direction of perfusion (see Table 2 legend).

It was decided to determine whether the periportal/perivenous gradient of ACC, ACL and FAS mass observed in normally feeding male rats was affected by nutritional conditions that favour either the repression or induction of lipogenesis. In male animals fasted for 48 h (low lipogenic rate), the periportal/perivenous gradient of each enzyme persisted. In fact, virtually all of the enzyme mass was located periportally (Fig. 6, compare lanes 1 and 5 with 2 and 6). The magnitude of the gradient could not be determined, since enzyme mass in

perivenous samples was generally too low to permit accurate quantification. After fasting (48 h) and refeeding (30 h) with a high-carbohydrate diet (high lipogenic rate), the periportal/perivenous gradient observed in fed and fasted animals was greatly decreased (Table 3 and Fig. 6, compare lanes 3 and 7 with 4 and 8). The apparent difference in band intensity between paired periportal and perivenous samples observed in the immunoblot reflects a larger load of released periportal protein. When the radioactivity associated with ACC, ACL and FAS was expressed per mg of released protein, the periportal/perivenous ratio of mass was decreased to approx. 1.0 for each enzyme (Table 3). These data indicate that the fold induction of ACC, ACL and FAS in perivenous hepatocytes during refeeding with a high-carbohydrate diet was greater than that in periportal hepatocytes in male rats. In contrast, the nutritional induction of ACC in female rats was similar in both periportal and perivenous hepatocytes (results not shown).

Since the zonal distributions of alanine aminotransferase, LDH and pyruvate kinase have been well characterized by using both microdissection and dual-digitonin-pulse perfusion, we have included them in the present study as reference enzymes. In agreement with previous studies [7,9,20,29,40,41], alanine aminotransferase and LDH were zoned periportally (Table 4). Alanine aminotransferase exhibited the largest gradient, having an average periportal/perivenous ratio of specific activity of 8.0, whereas the ratio for LDH was 3.0. The ratio for pyruvate kinase was 1.0.

The eluate yields (amount collected in total eluate expressed as a percentage of amount of activity or mg of protein in the liver at the start of the experiment) for alanine aminotransferase was $44.5 \pm 4.9\%$, for pyruvate kinase $38.8 \pm 4.8\%$, and for protein $18 \pm 3.5\%$ ($\pm$ S.E.M.; $n = 4$). The biopsy yields [% of the amount of activity or mg of protein ($\pm$ S.E.M.; $n = 4$) removed during perfusion] for alanine aminotransferase was $46.3 \pm 7.5\%$, for pyruvate kinase $45.3 \pm 1.2\%$, and for protein $26.0 \pm 6.5\%$. The reason for the lower yield of protein compared with

Table 2. ACC specific activity in periportal and perivenous eluates

Periportal (PP) and perivenous (PV) fractions were collected as described in the Experimental section. For both periportal and perivenous samples, the two fractions containing the highest amount of protein were pooled and centrifuged at 105 000 *g* for 60 min at 4 °C. ACC activity was measured in the supernatant immediately after centrifugation, and is expressed per mg of cytosolic protein and per mg of ACC-immunoreactive material as determined by quantitative immunoblotting using an IgG₁ monoclonal antibody (ACC.7) directed against ACC. Values shown are means $\pm$ S.E.M. of (*n*) separate experiments using male rats. Statistical significance (*P*) was assessed by Student's *t* test for paired data (ns, not significant). The initial perfusion direction was alternated for each experiment, so that in six experiments the initial direction was porta $\rightarrow$ cava (antegrade), and for five experiments the initial direction was cava $\rightarrow$ porta (retrograde). The activity gradient was unaffected by the initial direction of perfusion. For antegrade perfusion, the total activity gradient (calculated as the PP:PV ratio of munits/mg of protein at 10 mM-citrate in PP and PV eluates) was 2.5 ± 0.4 ; for retrograde perfusion, the PP:PV ratio was 3.3 ± 0.6 .

[Citrate] (mM)	Sp. activity (munits/mg of protein)		PP:PV	<i>P</i>
	PP	PV		
0	0.6 ± 0.1	0.2 ± 0.03	4.4 ± 1.5	< 0.01 (10)
0.5	1.5 ± 0.1	0.3 ± 0.03	5.0 ± 0.4	< 0.0001 (11)
10.0	1.7 ± 0.1	0.7 ± 0.08	2.8 ± 0.4	< 0.0001 (11)

	Sp. activity (units/mg of ACC)		PP:PV	
	PP	PV		
0	1.3 ± 0.3	0.5 ± 0.2	3.4 ± 1.4	< 0.01 (8)
0.5	2.4 ± 0.4	0.8 ± 0.2	3.6 ± 0.4	< 0.005 (8)
10.0	2.5 ± 0.4	1.6 ± 0.5	2.0 ± 0.4	ns (8)

the enzymes is due to the selective elution of cytosolic protein [28,39]. We were unable to determine in this study the fractional release of the lipogenic enzymes because of inaccuracies in the estimate of ACC and FAS activity in crude tissue extracts owing to competing reactions, and because of unacceptable non-specific background staining on immunoblotting.

DISCUSSION

Based on the release of protein from perfused rat liver by using the dual-digitonin-pulse-perfusion technique and its analysis by quantitative immunoblotting, the results of the present study indicate that three important enzymes of fatty acid biosynthesis, ACC, ACL and FAS, are present in increased concentrations in the periportal hepatocytes of the liver of the normally feeding male rat. This periportal/perivenous gradient persists in the livers of fasting rats, but is abolished on induction of lipogenesis with fasting followed by high-carbohydrate feeding.

Fig. 6. Alteration in periportal:perivenous mass gradient after refeeding with high-carbohydrate diet

Periportal and perivenous eluate fractions were obtained from male rats that were either fasted for 48 h (FA) or fasted 48 h and refed for 30 h with a high-carbohydrate chow (FR). Immunoblotting was performed with peak eluate fractions (pp2 and pv2). In preparation of the gel samples, the protein concentration of pp2 samples from each nutritional condition was adjusted to 5 mg/ml; the corresponding perivenous samples were then matched by an equal volume dilution. (a) Immunoblot with polyclonal antiserum directed against ACC. In our experience, ACC appears, on occasion, as multiple bands (even in purified preparations), likely reflecting proteolysis. (b) Blot with FAS/ACL antiserum. Lanes: 1, 3, 5, 7, pp2 (100 μ g of protein/lane); 2, 4, 6, 8, pv2 (40–75 μ g of protein/lane).

Table 3. Alteration in periportal:perivenous enzyme mass gradient after high-carbohydrate refeeding

Values represent the periportal:perivenous (PP:PV) ratio of c.p.m. of ¹²⁵I-Protein A associated with ACC, ACL and FAS per mg of eluted protein in peak fractions (pp2 and pv2) as determined by quantitative immunoblotting. Enzyme content in the peak periportal fraction was compared with that of the perivenous peak fraction from the same liver (fed males) by Student's *t* test (paired data), and results indicated a significant difference (**P* < 0.05, ***P* < 0.005). In samples from males that were fasted (48 h) and refed (30 h) with a high-carbohydrate diet, enzyme content in peak fractions did not differ significantly. A periportal:perivenous gradient of enzyme mass existed in samples from fasted (48 h) males (see Fig. 6), but the magnitude could not be determined, since the amount of enzyme in perivenous samples was generally below the limits of detection. Values shown are means $\pm$ S.E.M. for four separate experiments at each nutritional condition. The numbers of individual determinations are in parentheses.

Enzyme	PP:PV ratio in peak fractions		
	Fed (normal chow)	Fasted/refed (high-carbohydrate chow)	Fasted
ACC	1.9 ± 0.1 (8)	1.07 ± 0.2 (4)	> 1
ACL	1.4 ± 0.2 (10)	1.03 ± 0.1 (7)	> 1
FAS	3.1 ± 0.5 (10)	1.05 ± 0.1 (7)	> 1

These results suggest that the induction of the lipogenic enzymes was greater in the perivenous zone than in the periportal zone. No substantial intrahepatic gradients were observed in the female rat liver. These data do not

Table 4. Periportal and perivenous distributions of alanine aminotransferase, lactate dehydrogenase and pyruvate kinase

The periportal:perivenous (PP:PV) ratio of the specific activities (expressed as munits/mg of eluted protein) of alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) and pyruvate kinase (PK) was determined in each individual periportal and perivenous fraction from the four separate experiments depicted in Table 1. The PP:PV ratio in the right-hand column is the mean of the ratios of individual fractions. Values shown are the means $\pm$ S.E.M.

Eluate fraction ...	PP:PV ratio				Mean
	1	2	3	4	
ALAT	6.4 $\pm$ 1.2	10.9 $\pm$ 1.9	8.1 $\pm$ 1.2	6.4 $\pm$ 0.5	8.0
LDH	2.7 $\pm$ 0.3	2.5 $\pm$ 0.3	3.2 $\pm$ 0.2	3.4 $\pm$ 0.6	3.0
PK	1.0 $\pm$ 0.1	1.1 $\pm$ 0.1	0.9 $\pm$ 0.1	0.9 $\pm$ 0.1	1.0

necessarily indicate that the actual rate of fatty acid synthesis in periportal and perivenous hepatocytes differs, as this is also dependent in part on the supply of substrates and cofactors, which were not measured in this investigation.

The magnitude of the periportal/perivenous gradients measured by this technique has been conservatively corrected for the total released protein, since it has been shown that protein release from the periportal and perivenous zones differs with the dual-digitonin-pulse-perfusion technique in spite of an equal cytosolic protein concentration of periportal and perivenous cells [39]. However, previous work has shown that for the enzymes alanine aminotransferase, LDH and pyruvate kinase the amount of protein elution does not critically affect the periportal/perivenous specific-activity ratio [27]. We cannot ascertain with the present technique whether the percentages of the released enzymes from the periportal and perivenous perfusions were identical. Differential rates or magnitudes of release of both total protein and individual enzymes during the pulse perfusion could affect our estimation of the gradients, since we were unable to determine the fractional release of the lipogenic enzymes; in general, however, results obtained by the digitonin technique have agreed with those determined by microdissection for other enzymes. It will be important to confirm these observations by an alternative technique, and quantitative immunohistochemistry using monoclonal antibodies to each enzyme may provide additional evidence.

The periportal/perivenous-gradient values for the zonation of the enzymes alanine aminotransferase and LDH obtained in this study are slightly higher than values obtained with isolated hepatocytes. The minimal amount of digitonin utilized in this study affects only 15–25% of the cells in the upstream region of the sinusoid as determined by histological analysis (B. Quistorff & M. Roemert, unpublished work), resulting in a high degree of zonal selectivity. When hepatocytes are isolated by the digitonin/collagenase method [7,40], the procedure is, in essence, inverted, since in this case cells that are not destroyed by digitonin are those of interest. Therefore, if the zonal selectivity of the cell preparation

must be high, it is necessary to perfuse with larger amounts of digitonin. Since zonal selectivity decreases as the amount of digitonin used increases, the resultant cell preparation will also possess a substantially lower zonal purity [28]. Furthermore, in addition to zonal selectivity, a desired objective of hepatocyte preparation is high yield. This implies that the cell preparation represents a larger portion of the periportal/perivenous activity gradient of the particular enzyme, resulting in a smaller difference in enzyme specific activity between periportal and perivenous cells.

The data derived by using dual-digitonin-pulse perfusion with respect to the distribution of ACC and ACL conflict with data obtained by using microdissection of rat liver, which indicate a small gradient favouring the enhanced perivenous localization of ACC and ACL [21,22]. The resolution of this discrepancy is not readily apparent. With respect to ACC, enzyme activity in the microdissection study was measured after preincubation of the enzyme in the presence of Mg^{2+} and citrate [21]. This technique, although activating ACC, also leads to enzyme dephosphorylation and possibly proteolysis in crude extracts. It is possible that the heterogeneous distribution of protein phosphatase and/or proteinase activities could therefore alter the apparent distribution of ACC activity. No independent technique examining the distribution of ACC mass exclusive of ACC activity was reported in this study [21]. It is also possible that the rapidity and selectivity of the dual-digitonin-pulse-perfusion technique is better able to preserve the enzymes in their native state. Additionally, we have found a periportal gradient in male, but not in female, rats, whereas the previous studies found a small gradient to the perivenous region in both species.

Two types of periportal/perivenous gradients have been identified in the study of the lipogenic enzymes. In male rats, all three enzymes (ACC, ACL and FAS) exhibited a gradient in enzyme mass (Table 1), as judged by quantitative immunoblotting, implying an enhanced concentration of each in periportal hepatocytes. ACC also exhibited a periportal/perivenous gradient in actual specific activity, as determined by enzyme assay and immunoblotting (Tables 1 and 2). There are several possibilities that could account for the gradient in actual specific activity, including allosteric modulation, an alteration in covalent enzyme phosphorylation [42] or in the proportion of enzyme in the polymeric form [43], or a combination of these. The periportal gradient of ACC specific activity was more pronounced in the absence of, or at low concentrations, of citrate, an allosteric activator of ACC. Interestingly, a difference in ACC activity, characterized by the periportal enzyme possessing a significantly lower activation constant for citrate compared with the enzyme isolated from perivenous hepatocytes, persists after enzyme isolation by avidin-Sepharose chromatography (J. L. Evans, B. Quistorff & L. A. Witters, unpublished work). This finding argues against allosteric modulation, and suggests that a difference in the degree of phosphorylation may be responsible for the gradient in ACC activity. This result may prove particularly important in delineating the details of the activity gradient, in light of previous reports indicating that the dephosphorylation of ACC renders it relatively citrate-independent [44,45]. The present observations would suggest that the periportal enzyme is dephosphorylated relative to the perivenous

enzyme. Experiments designed to measure the phosphate content of ACC isolated from periportal and perivenous eluates, along with comparing protein kinase and phosphatase activities in the eluates, will be useful.

The mechanism(s) underlying the creation of these intrahepatic gradients for the lipogenic enzymes is of obvious interest (for reviews, see [2] and [20]). Katz and co-workers have reported that both insulin and glucose can lead to the induction of ACC and ACL in primary cultures of rat hepatocytes [46,47]. Periportal hepatocytes are exposed to higher concentrations of both insulin and perhaps glucose arising from the splanchnic circulation. Other hormones, such as glucagon, catecholamines and corticosteroids, are also degraded during passage through the liver. It is possible that the higher concentrations of these substances or other hormones and metabolites might maintain a periportal/perivenous gradient of the lipogenic enzymes. Alternatively, it is possible that metabolites released from periportal cells (with an increasing concentration gradient to the perivenous side) might suppress the expression of the lipogenic enzymes in the perivenous cells. From the present study, it is noteworthy that high-carbohydrate feeding, which is associated with increasing insulin and carbohydrate delivery to the liver, in fact leads to a greater fold induction of lipogenic enzyme content in the perivenous cells of male rats. The differences between male and female rats suggest that gonadal steroids may play a role in establishing intrahepatic gradients.

The periportal/perivenous gradient in ACC specific activity might also be influenced by the hormonal milieu perfusing the liver. Insulin has been shown to lead to the activation and dephosphorylation of ACC, associated with increased citrate-independence and a decrease in the activation constant for citrate in rat hepatoma cells [48], and to increase the proportion of ACC present as an activated enzyme polymer [49]. Relative increases in the periportal insulin concentration might therefore be responsible for this observed gradient, which is independent of enzyme mass.

The potential significance of the observed intrahepatic gradients of the enzymes of fatty acid biosynthesis demonstrated in the present study and of 3-hydroxy-3-methylglutaryl-CoA reductase [25,26] remains to be determined. It is possible that the sites of plasma lipoprotein synthesis and/or degradation within the liver are differentially segregated as either a result or a consequence of hepatic zonation. In addition, since malonyl-CoA, the product of ACC, is believed to play an important role in the regulation of ketogenesis from fatty acids [50], differential distribution of ACC activity could regulate the rates of ketone production from perivenous and periportal cells.

Note added in proof (received 13 March 1989)

It has recently been reported that liver fatty acid-binding protein (L-FABP) is zoned periportal in the hepatic acinus of male rats [51]. The ability of L-FABP to compete for fatty acyl-CoA, thereby protecting ACC from the inhibitory effects of these molecules [52], may be a contributory component to the periportal specific-activity gradient for ACC found in the present investigation.

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