The fuel of the spleen

Studies using a new method for perfusing the rat spleen with whole blood

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1. An improved rat spleen perfusion is described incorporating a method of defibrination which avoids the use of heparin and enables the spleen to be perfused with rat blood for several hours at a haematocrit of 40 % and for 12 h or more at a haematocrit of 20 %. 2. Glucose oxidation accounted for 11.6 % of the total oxygen consumption but this represented only 8 % of total glucose uptake, which was largely converted to lactate and released into the perfusate. However, significant amounts of lactate were oxidized. These results can be explained by the presence of at least two cell populations, one emphasizing the anaerobic oxidation of glucose and the other aerobic metabolism, particularly of lactate. 3. Non-esterified fatty acid and 3-hydroxybutyrate, when available at physiological concentrations, were shown to be major oxidative fuels of the spleen. 4. Chylomicron triacylglycerol was hydrolysed readily and taken up, but not oxidized extensively.

INTRODUCTION

Isolated intact perfused organs have several physiological advantages over more disrupted systems when used for metabolic studies. In particular, when a blood perfusate is employed having a physiological haematocrit value, this allows an optimal O_2 consumption to occur at a normal rate of flow. In turn, this permits substrates and products to be provided and removed at concentrations and rates closely reflecting those *in vivo*. In the present study, a system has been developed which enables perfusion of the spleen for several hours at 40 % haematocrit and for at least 12 h at 20 % haematocrit. Perfusion of the spleen with media containing erythrocytes presents an additional difficulty, compared with perfusion of other organs, because erythrocytes, easily damaged by methods used to prepare the perfusate and by the perfusion apparatus itself, are sequestered by the spleen, one of its major functions *in vivo*. When excessive, this leads to obstruction and resistance preventing the maintenance of an adequate flow rate. The use of heparin, to prevent coagulation, minimizes damage to cells but has the potential for activating and releasing lipoprotein lipase which would cause degradation of lipoproteins and release of non-esterified fatty acids (NEFA). For these reasons it was necessary to avoid the use of heparin in perfusate preparation, as lipoprotein lipase has been reported to be present in spleen (Korn, 1955; Krebs, 1972; Mindham & Mayes, 1989). We therefore developed an alternative method utilizing defibrination which also minimized cell damage.

The perfused spleen has been utilized for investigations in the rat (Ford & Gowans, 1967; Ford, 1969), pig and human (Pabst & Trepel, 1974), where it has been used for the study of lymphocyte migration. However these studies employed heparinized, low haematocrit or erythrocyte-free perfusates and revealed little information on spleen metabolism. We now report details of an improved

perfusion system and results of a study to determine the major fuels and products of spleen metabolism. The system has been developed with the ultimate object of investigating reverse cholesterol transport (Mindham *et al.*, 1988). The utilization and oxidation of glucose, lactate, oleate, 3-hydroxybutyrate and chylomicrons are described using ^{14}C -labelled substrates. These studies have been reported in preliminary form (Mindham *et al.*, 1987).

EXPERIMENTAL

Animals and materials

Male Wistar rats were used as spleen donors (350–370 g body wt.) and as blood donors (350–450 g body wt.). They were fed on a standard pelleted diet and kept under constant daylength (12 h) and temperature (25 °C). D-[U- ^{14}C]Glucose, sodium L(+)-[U- ^{14}C]lactate, [1- ^{14}C]oleic acid, [1- ^{14}C]palmitic acid and sodium D(–)-3-hydroxy[3- ^{14}C]butyrate were obtained from Amersham International, Amersham, Bucks., U.K. Oleic acid, sodium pentobarbital and antibiotics were obtained from Sigma Chemical Co., Poole, Dorset, U.K., and sodium D(–)-3-hydroxybutyrate was from Aldrich Chemical Co., Gillingham, Dorset, U.K. All enzymes were obtained from Boehringer Corp., Lewes, E. Sussex, U.K.

Perfusion apparatus

A diagram of the perfusion apparatus is shown in Fig. 1. Surgical grade plastic tubing and connectors (Portex Ltd, Hythe, Kent, U.K.) were used throughout, except for tubing in contact with pumps where silicone rubber tubing (Esco Rubber; Sterilin Ltd, Hounslow, Middlesex, U.K.) was used. The spleen was placed in a glass bowl (Aimer Products, Enfield, Middlesex, U.K.) maintained at 37 °C by a water jacket fed from a circulating water bath. A pump ('Hiloflow'; Metering Pumps Ltd.,

Abbreviation used: NEFA, non-esterified fatty acids.

Fig. 1. Apparatus for perfusing the isolated rat spleen

A, air bubble injected into graduated flowmeter (FM); BT, bubble trap; B, water-jacketed bowl; C₁, C₂, stop-cocks for sampling; CF, clingfilm; E₁, E₂, oxygen electrodes; F, cotton poplin filter; H, humidifier; I, substrate infusion; PE, pressure equalizer; PT, moist paper tissue; P₁, P₂, blood pumps; S, spleen; ST, stirrer; T₁, T₂, thermistor-thermometer.

London, U.K.), causing minimal haemolysis, circulated blood from the main reservoir via an oxygen electrode and back to the reservoir. The pO_2 values of both the arterial and venous blood were measured with E5047 oxygen electrodes (Radiometer, Copenhagen, Denmark). Blood was circulated by a variable flow rate peristaltic pump (Varioperspex 12000; LKB, Bromma, Sweden) to the spleen via a cotton poplin filter and bubble trap. A narrow-bore standpipe indicated the pressure required to maintain flow. A mixture of humidified O_2/CO_2 (19:1 and air/ CO_2 (19:1) was circulated through the apparatus and passed over the blood in the reservoir, and the proportions adjusted to maintain arterial pO_2 at 100 mmHg (13.3 kPa). The gas mixture was withdrawn from the reservoir by a moderate vacuum and CO_2 was absorbed by bubbling through two columns of 2 M-NaOH connected in series. The reservoir blood was kept stirred and the perfusion circuit was housed in a thermostatically controlled cabinet maintained at 37 °C. Thermistor probes (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) were placed adjacent to the spleen and inside the cabinet to monitor temperature.

Perfusate

Blood was collected from the iliac bifurcation of the aorta of rats anaesthetized with diethyl ether, using

EDTA as anticoagulant (2.5 mg/ml of blood). The blood was cooled in ice and the plasma removed by centrifugation at 3000 *g* for 20 min at 4 °C. The plasma was recalcified by the addition of excess $CaCl_2$ to give a concentration of 10 mM, which allowed clotting to occur when incubated at 37 °C for 20 min. The precipitated fibrin was removed by centrifugation at 3000 *g* for 20 min at 4 °C. The resulting serum was dialysed against a modified Krebs-Henseleit (1932) bicarbonate buffer for 20 h at 2 °C to remove vasoconstrictor factors (Mayes & Felts, 1966). The buffer contained glucose (250 mg/dl), plasma amino acids (67 mg/dl), and half the specified quantity of calcium. Blood was reconstituted by mixing erythrocytes, washed with 0.9% NaCl, with the dialysed serum to give a haematocrit of 30%. Ampicillin (sodium salt) and streptomycin sulphate at concentrations of 5 mg/dl and 10 mg/dl of blood respectively were added. The volume of blood perfusate used in each perfusion was approx. 50 ml.

Preparation of the isolated spleen

Rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt.) dissolved in 0.15 M-NaCl and the abdomen opened by a median and transverse incision. Loose ligatures were placed around the portal vein above and below its junction with the

splenic vein. The spleen was mobilized by section of the gastrosplenic and other ligaments attaching it to adjacent viscera. Vessels connecting the splenic artery and vein to the pancreas were ligated and the pancreas was cut away. The right gastric and hepatic arteries were ligated, followed by the aorta above and below the coeliacomesenteric artery. Immediately, this last-named artery was cannulated from the aorta using a stainless steel cannula (external diam. 1.07 mm). The spleen was flushed free of blood with the modified Krebs–Henseleit buffer (25–30 °C), which was further modified to contain 10 mM- K^+ and 3 g of bovine serum albumin/100 ml (Fraction 5; ICN Biomedical, High Wycombe, Bucks., U.K.) and equilibrated with O_2/CO_2 (19:1). The hepatic portal vein was severed rapidly both above and below the loose ligatures to permit a free flow through the spleen. A metal cannula (external diam. 1.8 mm) filled with 0.9% NaCl containing 200 units of heparin/ml was then tied into the portal vein near its junction with the splenic vein, using one of the loose ligatures. The other loose ligature was tied and the spleen was cut free from the animal, placed in the glass bowl and washed with a saline solution containing ampicillin (5 mg/dl) and streptomycin sulphate (10 mg/dl) before being connected into the perfusion circuit. The splenic flow was interrupted for only a very short time during aortic cannulation and during transfer to the perfusion cabinet. The total time for establishing the perfusion averaged 30 min and throughout this time the spleen was kept moist with saline and handled as little as possible. The spleen was perfused at a flow rate of 0.75 ml/min, i.e. approx. 1 ml/min per g.

Preparation of labelled chylomicrons and NEFA

Chylomicrons labelled in the triacylglycerol fatty acid moiety were prepared from rats fed 0.5 ml of corn oil by intubation. After 1–2 h the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt.) and the thoracic duct cannulated with polyethylene tubing (external diam. 1.52 mm) according to Bollman *et al.* (1948). [^{14}C]Palmitic acid (50 μ Ci), neutralized with KOH and emulsified with sodium taurocholate, was injected through the stomach wall into the pyloric antrum. The collected lymph was layered under NaCl (density 1.006 g/ml) in 6.5 ml tubes and centrifuged for 6×10^5 g $\cdot$ min ($r_{av.} = 64.2$ mm) in a fixed-angle rotor at 12 °C. The top fraction was harvested and used at once.

Labelled NEFA were prepared by dissolving 20 mg of oleic acid labelled with [^{14}C]oleic acid (5 μ Ci) in 0.5 ml of 0.12 M-NaOH and added with stirring to 1 ml of 15% bovine serum albumin at 40 °C.

Analytical methods

Perfusate glucose was determined by a glucose oxidase method (Boehringer Corp.). L-(+)-Lactate and pyruvate were determined by the method of Hohorst (1963). D-(–)-3-Hydroxybutyrate and acetoacetate were determined by the method of Williamson *et al.* (1962). NEFA were determined by microtitration based on the method of Trout *et al.* (1960). Triacylglycerol was estimated by the method of Carlson (1959).

After perfusion, spleens were weighed and homogenized with 70 ml of chloroform/methanol (2:1, v/v), extracted overnight at room temperature, filtered and partitioned with 0.4 vol. of 0.03 M-HCl. Portions of the

chloroform extracts were dried under N_2 and separated into lipid classes by t.l.c. using silica gel G and hexane/diethyl ether/formic acid (80:20:2, by vol.) as the developing solvent. After location of the bands with iodine, they were transferred into scintillation vials for counting. Phospholipids were extracted from the gel with chloroform/ethanol/water/acetic acid (30:100:20:2, by vol.), and portions of the extract were blown down in vials for counting.

Potassium and sodium in perfusate serum were determined by flame photometry. O_2 consumption was calculated from the flow rate and difference in arterial–venous O_2 content. The O_2 content of the blood was measured using a Mk.2 Blood Micro System (Radiometer, Copenhagen, Denmark) after displacement into solution by potassium ferricyanide, based on a method by Solymar *et al.* (1971).

Radioactivity was measured using a Packard liquid scintillation counter (model 4530). Counts were corrected for efficiency using an external standard and appropriate quench standards. Dried lipid extracts and silica gel bands from t.l.c. were counted for radioactivity in 12 ml of toluene containing 0.45% (w/v) 2,5-diphenyloxazole and 0.038% (w/v) 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Packard Instrument Co., Reading, U.K.) and 6 ml of toluene/ethanol/acetic acid (1160:40:1, by vol.). In experiments utilizing radioactive glucose, samples were deproteinized with $HClO_4$ and purified on miniature columns containing DEAE-Sephacel (Pharmacia, Milton Keynes, U.K.) equilibrated with 0.01 M-Tris buffer, pH 8.5. Bound material (primarily lactate formed by the erythrocytes and spleen) was eluted with 1 M-Tris buffer, pH 8.5. Column effluents (4 ml) were counted with 6 ml of Instagel (Packard Instrument Co.) In the experiments with radioactive lactate and oleic acid, small samples of deproteinized serum or serum respectively were made up to 1 ml with water and counted with 10 ml of Optiphase X (Fisons, Ipswich, Suffolk, U.K.).

Oxidation of test metabolites to CO_2 was measured by the arterial–venous difference in radioactivity liberated from the blood perfusate. Blood (0.5 ml) was added to the outer compartment of a 50 ml centre-well flask, containing 5 ml of methanol and 2 ml of water, to which H_2SO_4 was added. The CO_2 evolved was trapped in 0.5 ml of Hyamine hydroxide (B.D.H., Poole, Dorset, U.K.) in methanol in the centre-well and counted with 6 ml of ethanol and 12 ml of toluene scintillant. Radioactive ketone bodies were determined by a modification of the method of Mayes & Felts (1967) using aniline citrate at 30 °C to decarboxylate acetoacetate.

RESULTS

Viability of the perfused rat spleen

Physical, physiological and biochemical criteria may be used to assess the viability of perfused organs. The appearance of the spleen, the perfusate flow rate, the resistance to flow as measured by the inflow pressure, potassium loss into the perfusate and O_2 consumption were all used in evaluating the present preparation. The gross appearance of the spleen perfused with a blood medium was similar to that *in vivo*, i.e. a uniform dark red colour.

In a series of trial perfusions it was shown that the preparation of the blood perfusate had a significant

effect on the development of spleen resistance (Fig. 2). Defibrination by stirring with wooden applicator sticks resulted in a rapid and sustained rise in perfusion pressure making it impossible to maintain the desired flow rate of 1 ml/min per g, the presence of a haematocrit of 40% being considerably more deleterious than one of 20%. In contrast, the use of EDTA to prevent coagulation in the presence of erythrocytes, followed by removal of fibrinogen from the separated plasma with excess Ca^{2+} , and final reconstitution of the blood using washed erythrocytes, resulted in a markedly reduced rise in spleen resistance. Under these conditions, an adequate flow rate could be maintained for several hours at full haematocrit and for at least 12 h at 20% haematocrit. In practice, we have adopted a haematocrit of 30% for all perfusions lasting up to 5 h.

In the perfused rat liver, potassium efflux is one of the most sensitive indicators of decreased tissue function (Ruderman & Herrera, 1968). It is released during tissue damage, anoxia and when the tissue is cooled. An efflux of potassium was observed from spleens during their isolation when flushed free of blood with Krebs–Henseleit bicarbonate buffer at 25–30 °C. The K^+ concentration of the buffer was increased to prevent this. During perfusion with blood perfusates the serum potassium remained constant at a mean value of 6.16 ± 0.03 mM (mean $\pm$ S.E.M., $n = 5$). In addition to the spleen, damaged erythrocytes are also a potential source of serum potassium.

Oxygen electrodes were placed in the perfusate inflow and outflow to monitor differences in $p\text{O}_2$ on a continuous basis (Fig. 1). The O_2 consumption of the perfused spleen, as calculated from the difference in oxygen content of arterial and venous blood, indicated constant O_2 utilization by the spleen throughout the period of perfusion. There was no significant gain in weight of the spleen during perfusion, indicating lack of oedema.

Metabolic studies

Six groups, each comprising at least four perfusions of 2 h duration, were carried out. Each group enabled the study of the metabolism of one labelled substrate added to a blood perfusate of basal composition. The basal composition at the start of the experimental period resulted from the dialysis of the blood perfusate against the modified Krebs–Henseleit bicarbonate buffer and to circulation in the apparatus prior to the experiment. It comprised erythrocytes at a haematocrit value of 30%, defibrinated rat serum containing all the plasma amino acids at appropriate concentrations, glucose at a concentration of $7.2 \mu\text{mol/ml}$ of blood, lactate at a concentration of $1.8 \mu\text{mol/ml}$ of blood and NEFA at a concentration of $0.52 \mu\text{mol/ml}$ of serum. L(+)-Lactate was negligible in the freshly dialysed perfusate but increased in concentration prior to the start of the experiment due to erythrocyte glycolysis and during perfusion due to production from both spleen and erythrocytes. A total of $5 \mu\text{Ci}$ of added substrate was used per perfusion except in the case of chylomicrons which were labelled *in vivo* and varied in total radioactivity between $3.5 \mu\text{Ci}$ and $6 \mu\text{Ci}$.

Small vestiges of pancreatic and adipose tissue surrounding the splenic artery and vein were unavoidably perfused in addition to the spleen. As these would contribute to metabolism, their effect was measured by continuing the perfusion at the same inflow pressure for a further hour after the spleen had been carefully removed, following close ligation of its afferent and efferent vessels. A correction for the metabolic contribution of this residual tissue was made in calculating the results. In general, this was relatively small and averaged 18%.

Carbohydrate metabolism

The mean glucose concentration decreased during the

Fig. 2. Effect of method of perfusate preparation and haematocrit on resistance to blood flow of the perfused spleen

Spleens were perfused with blood prepared by two methods of defibrination: (i) by stirring whole blood with applicator sticks or (ii) by removal of fibrinogen from plasma with excess Ca^{2+} (see the text). $\square$, Defibrination by stirring, 40% haematocrit; $\blacksquare$, defibrination by stirring, 20% haematocrit; $\circ$, defibrination with Ca^{2+} , 40% haematocrit; $\bullet$, defibrination with Ca^{2+} , 20% haematocrit. The number of perfusions is shown in parentheses.

Table 1. Metabolism of glucose, L(+)-lactate and D(-)-3-hydroxybutyrate by perfused rat spleen

Spleens were perfused for 2 h (see text) with ^{14}C -labelled substrate ($5\ \mu\text{Ci}$) at the concentrations indicated. Values are the means $\pm$ S.E.M. from the number of experiments shown in parentheses. NA, not applicable; *, net output; †, basal perfusate (see text).

$[^{14}\text{C}]$ Substrate	Substrate concentration ($\mu\text{mol/ml}$ of blood)		Net uptake ($\mu\text{mol/h}$ per g)	Incorporation ($\mu\text{mol/h}$ per g) into:				
	Initial	Final		CO_2	Spleen diacylglycerols	Spleen triacylglycerols	Lactate	Acetoacetate
[U- ^{14}C]Glucose (6)	7.2†	4.1†	20.0 ± 1.5	1.64 ± 0.19	0.009 ± 0.002	0.014 ± 0.002	14.6 ± 2.0	NA
[U- ^{14}C]L(+)-Lactate (6)	1.8†	7.5†	$-29.3 \pm 4.0^*$	6.15 ± 0.91	0.012 ± 0.003	0.019 ± 0.007	NA	NA
D(-)-3-Hydroxy-[3- ^{14}C]butyrate (4)	2.4	2.3	4.11 ± 0.26	2.26 ± 0.23	0.0 ± 0.0	0.011 ± 0.002	NA	1.05 ± 0.15

perfusions from 7.2 to 4.1 $\mu\text{mol/ml}$ of blood. Determination of the arterial-venous difference in glucose concentration indicated a mean net uptake of 20.0 $\mu\text{mol/h}$ per g (Table 1). Oxidation of glucose to CO_2 was calculated from the difference in radioactive bicarbonate concentration across the spleen. It was surprisingly small (1.6 $\mu\text{mol/h}$ per g) and was constant throughout the period of perfusion despite the large increase in lactate concentration. It represented only 8% of the total glucose uptake, the bulk of the uptake being released primarily as lactate. Lactate determination demonstrated a net mean release of 29.3 $\mu\text{mol/h}$ per g. The mean lactate concentration increased during the perfusions from 1.8 to 7.5 $\mu\text{mol/ml}$ of blood. Mean oxidation of lactate to CO_2 was found to be 6.2 $\mu\text{mol/h}$ per g, i.e. greater than that of glucose. In addition, oxidation of lactate, unlike the other substrates, did not reach a stable rate but continued to increase as the perfusate lactate concentration increased due to the continuing production by the spleen and erythrocytes throughout the time of perfusion

Fig. 3. Effect of perfusate L(+)-lactate concentration on oxidation of L(+)-lactate to CO_2 in the perfused rat spleen

Values are from six perfusions with addition of $5\ \mu\text{Ci}$ of L(+)-lactate as described in the text. A minimum of three values from each perfusion is shown, indicating lactate oxidation at 0–30, 30–60, 60–90 and 90–120 min. The regression line is highly significant ($P < 0.001$).

(Fig. 3). Glucose and lactate concentrations, and consequently their uptake and release were unaffected by addition and oxidation of other substrates such as chylomicrons, oleic acid and D(-)-3-hydroxybutyrate. Small amounts of radioactivity originating in glucose and lactate were found in triacylglycerols and diacylglycerols upon lipid analysis of the spleen (Table 1). Pyruvate estimation indicated a net production by the spleen of $4.63 \pm 0.63\ \mu\text{mol/h}$ per g.

Ketone body metabolism

In many tissues ketone bodies, when present, have been shown to be major oxidative fuels. In a group of four experiments (Table 1), D(-)-3-hydroxy[3- ^{14}C]butyrate was added to the perfusate at a perfusate concentration of 2.38 $\mu\text{mol/ml}$. Mean uptake was $4.11 \pm 0.26\ \mu\text{mol/h}$ per g, with $2.26 \pm 0.23\ \mu\text{mol/h}$ per g oxidized to $^{14}\text{CO}_2$ and $1.05 \pm 0.15\ \mu\text{mol/h}$ per g converted to and released as acetoacetate. Lipid analysis of spleen extracts revealed small amounts of radioactive hydroxybutyrate carbon incorporated into splenic glycerols.

NEFA metabolism

NEFA metabolism by the spleen was examined at two concentrations, 1.74 $\mu\text{mol/ml}$ and 0.52 $\mu\text{mol/ml}$ of serum, the latter being the basal concentration. In both cases the radioactive oleic acid dose, with or without added mass complexed to bovine serum albumin, was added slowly (over 4 min) to the perfusate. At both concentrations there was substantial esterification to triacylglycerol and phospholipid and oxidation to CO_2 (Table 2). The balance between the pathways of esterification and oxidation was significantly different at the two concentrations tested. At increased NEFA concentration the proportion of the uptake oxidized decreased while the proportion esterified, particularly to triacylglycerol, increased.

Chylomicron metabolism

Upon analysis, the lymph chylomicrons labelled with [1- ^{14}C]palmitate *in vivo* gave a major triacylglycerol fraction containing 94% of the radioactivity and the remaining radioactivity was equally distributed between fatty acid and phospholipid. At a perfusate concentration

Table 2. The metabolism of NEFA and chylomicrons in perfused rat spleen

Spleens were perfused for 2 h with ^{14}C -labelled substrate ($5\ \mu\text{Ci}$) at the concentration given. Analysis of lipid classes was determined by t.l.c. of chloroform extracts of spleen or serum and subsequent scintillation counting (see the Experimental section). Values are means $\pm$ S.E.M. ($n = 4$). NA, not applicable; *, basal perfusate (see text).

^{14}C Substrate	Substrate concentration ($\mu\text{mol/ml}$ of serum)		Net uptake ($\mu\text{mol/h}$ per g)	Incorporation ($\mu\text{mol/h}$ per g) into:			
	Initial	Final		CO_2	Spleen phospholipids	Spleen triacylglycerols	NEFA released into perfusate
NEFA[$1\text{-}^{14}\text{C}$]oleate	0.52*	0.46*	1.38 ± 0.15	0.61 ± 0.09	0.55 ± 0.12	0.10 ± 0.01	NA
NEFA[$1\text{-}^{14}\text{C}$]oleate	1.74	1.67	2.54 ± 0.08	0.87 ± 0.07	1.03 ± 0.14	0.57 ± 0.07	NA
[$1\text{-}^{14}\text{C}$]Palmitoyl triacylglycerol (as chylomicrons)	1.90	1.85	0.88 ± 0.07	0.07 ± 0.00	0.29 ± 0.05	0.45 ± 0.04	0.47 ± 0.06

Table 3. Oxidation of metabolic substrates by the perfused rat spleen

Spleens were perfused for 2 h with ^{14}C -labelled substrate at the concentrations indicated. Oxidation of substrate and total O_2 consumption were determined by arterial-venous difference in $\text{H}^{14}\text{CO}_3^-$ and O_2 content respectively. Values are the means $\pm$ S.E.M. from the numbers of experiments shown in parenthesis. †, Not measured directly; data from line 4 were used as measured under the same conditions.

^{14}C Substrate	Substrate concentration ($\mu\text{mol/ml}$ of blood or * $\mu\text{mol/ml}$ of serum)		Oxidation to CO_2 ($\mu\text{mol/h}$ per g)	Uptake oxidised to CO_2 (%)	O_2 requirement ($\mu\text{mol/h}$ per g)	Total O_2 consumption ($\mu\text{mol/h}$ per g)	O_2 requirement as % of total O_2 consumption
	Initial	Final					
[U- ^{14}C]Glucose (6)	7.2	4.1	1.64 ± 0.19	8.2	9.81	$81.6 \pm 7.2^\dagger$	12.0
[U- ^{14}C]L(+)-Lactate (6)	1.8	7.5	6.15 ± 0.91	NA	18.44	$81.6 \pm 7.2^\dagger$	22.6
D(-)-3-Hydroxy[$3\text{-}^{14}\text{C}$]-butyrate (4)	2.4	2.3	2.26 ± 0.23	55.0	10.17	88.8 ± 8.1	11.5
NEFA[$1\text{-}^{14}\text{C}$]oleate (4)	0.52*	0.46*	0.61 ± 0.09	44.3	15.61	81.6 ± 7.2	19.1
NEFA[$1\text{-}^{14}\text{C}$]oleate (4)	1.74*	1.67*	0.87 ± 0.07	34.1	22.12	81.0 ± 3.9	27.3
[$1\text{-}^{14}\text{C}$]Palmitoyl triacylglycerol (as chylomicrons) (4)	1.90*	1.85*	0.07 ± 0.003	8.1	5.43	87.6 ± 9.0	6.2

of $1.9\ \mu\text{mol}$ of triacylglycerol/ml of serum, 8% of the uptake into the spleen was oxidized to CO_2 but most of the uptake was deposited in triacylglycerols and phospholipids (Table 2). Unlike NEFA, the pattern of deposition in esterified products favoured triacylglycerol rather than phospholipid. However, any incomplete washout of labelled chylomicrons from the spleen at the termination of perfusion may have contributed to this result. In addition, and most significantly, $0.47\ \mu\text{mol/h}$ per g of spleen of chylomicron triacylglycerol was hydrolysed by the spleen and released as NEFA to the perfusate.

Oxidative metabolism

As previously stated, O_2 consumption was constant throughout the perfusions. A mean value of $1.41 \pm 0.05\ \mu\text{mol}$ of O_2/min per g was obtained for 21 perfusions. The oxidation of the metabolic substrates studied in this investigation is summarized in Table 3. The greatest proportion of the uptake oxidized to CO_2

was found in the case of D(-)-3-hydroxybutyrate and with NEFA. With respect to the basal perfusate which contained mean concentrations of glucose, lactate and NEFA of $5.8\ \mu\text{mol}$ and $4.5\ \mu\text{mol/ml}$ of blood and $0.52\ \mu\text{mol/ml}$ of serum respectively, over 50% of the total O_2 consumption could be accounted for by the oxidation of these three substrates. Of further interest was the fact that O_2 consumption did not change significantly when a higher concentration of NEFA was used, or addition of D(-)-3-hydroxybutyrate or chylomicrons was made to the basal perfusate, even though the additions themselves accounted for a significant share of the overall O_2 consumption.

DISCUSSION

The spleen is one of few tissues on which quantitative information on the fuels of respiration is lacking. There are some data on oxygen and glucose utilization and

lactate production in pig spleen perfused with haemoglobin-free perfusates (Pabst & Trepel, 1974). However, these perfusions are likely to be oxygen deficient and this is reflected in a much lower oxygen consumption and higher lactate production than obtained in our studies on rat spleen. However, similar values to the present have been found for glucose uptake and lactate release from direct measurements of arterial-venous difference in humans (Vaupel *et al.*, 1977). The oxygen consumption of the perfused rat spleen ($1.41 \mu\text{mol of O}_2/\text{min per g}$) compares favourably with other tissues but is less than that of $3.6 \mu\text{mol of O}_2/\text{min per g}$ reported for the blood-perfused liver (Laker & Mayes, 1982).

Although a large uptake of glucose was observed, only a small proportion was oxidized to CO_2 , the remainder being released primarily as lactate and pyruvate. The ratio of lactate to pyruvate released by the preparation was 7.7:1, indicating a physiologically satisfactory intracellular redox state and state of oxygenation. A substantial oxidation of lactate was observed particularly as the concentration increased. The extent to which lactate is oxidized at decreased concentrations ($< 1 \mu\text{mol/ml}$) was not determined. However, the spleen seems similar to some other tissues which, at elevated concentrations (up to $10 \mu\text{mol/ml}$) such as occur during exercise, increasingly oxidize lactate, e.g. heart muscle, liver, kidney, brain and testes (Krebs, 1972). Our results infer the presence of active lactate and pyruvate dehydrogenase in the spleen. The results reporting low oxidation of glucose to CO_2 with marked conversion to lactate on the one hand compared with the greater oxidation of lactate to CO_2 on the other, in the presence of similar concentrations of glucose and lactate, are inconsistent with a single population of cells having a homogeneous pyruvate pool, and indicate the presence of at least two cell populations. This agrees with histological studies of the spleen which show several cell populations, e.g. red pulp and white pulp. One of these cell types, which must predominate, is a net producer of lactate from glucose and emphasizes glycolytic metabolism having a poor capacity for aerobic metabolism of glucose, and therefore of pyruvate. Other cells take up lactate and oxidize it to CO_2 , having greater aerobic capacity for the oxidation of pyruvate.

Ketone bodies have been shown to be major fuels of oxidative metabolism in the whole organism (Wick & Drury, 1941; Krebs, 1961) and in individual organs such as the heart (Williamson & Krebs, 1961). In the current experiments, D(-)-3-hydroxybutyrate was supplied to the spleen at a physiological concentration of $2.4 \mu\text{mol/ml}$ and substantial oxidation was observed, indicating that ketone bodies, if present in significant concentrations, are also major fuels of the spleen. The marked production of acetoacetate upon addition of D(-)-3-hydroxybutyrate demonstrates the presence of active D(-)-3-hydroxybutyrate dehydrogenase in the spleen and also testifies to an intramitochondrial redox state indicative of adequate oxygenation of the preparation. Results using the perfused spleen in the presence of low and increased levels of NEFA indicate NEFA to be an important fuel source for the spleen as they are readily oxidized to CO_2 . In addition, they are an important source of esterified lipid in the form of triacylglycerol and phospholipid. Active uptake and metabolism of labelled chylomicrons by the spleen was

indicated by a substantial incorporation of label into CO_2 , spleen triacylglycerol and phospholipids. This was probably mediated by the presence of lipoprotein lipase since the metabolism of chylomicrons resulted in a substantial release of NEFA to the perfusate which quantitatively amounted to about half of the quantity of triacylglycerol fatty acid that was esterified and oxidized by the spleen. If this represents the situation *in vivo* it is implied that NEFA released by the spleen will pass into the portal vein and thence be directed to the liver where they will augment NEFA released from the remainder of the splanchnic bed. We have confirmed by studies on acetone powders of the spleen that there is more than sufficient lipoprotein lipase present in this organ to account for chylomicron metabolism (Mindham & Mayes, 1989).

Present investigations of the oxidative capacity of the spleen have shown an ability of this organ to take up and oxidize major substrates that have been found to provide the fuel of mammalian tissues, i.e. glucose, lactate, NEFA, ketone bodies and chylomicrons. Moreover, the basal perfusate consisting of dialysed, defibrinated whole blood containing glucose, lactate and NEFA, as well as a full complement of plasma amino acids, was shown to be adequate for maintaining maximum oxygen consumption of the spleen. Thus, addition of extra NEFA, D(-)-3-hydroxybutyrate or chylomicrons to this perfusate did not promote extra oxygen utilization in spite of the fact that each of the added substrates was oxidized actively to CO_2 and accounted for a significant proportion of the total oxygen consumption. It is therefore implied that energy metabolism of the spleen is closely regulated, as the oxidation of one or more of the available substrates must have been inhibited upon addition of extra lactate, NEFA or D(-)-3-hydroxybutyrate. As we did not investigate which of the available substrates' oxidation was inhibited, it is not possible to comment on the mechanisms involved. However, it would appear that, as in some other tissues, NEFA, ketone bodies and lactate are used preferentially when available.

Throughout the period of perfusion the appearance of the spleen was indistinguishable from that *in vivo* and the measured biochemical parameters of spleen metabolism, including oxygen consumption, remained constant. The proposed technique is likely to be physiologically valid and a useful tool for studying spleen metabolism. We have recently employed it in the study of reverse cholesterol transport (Mindham *et al.*, 1988).

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