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Regulation of Carbohydrate Metabolism in Lymphoid Tissue

QUANTITATIVE ASPECTS OF [U-^{14}C]GLUCOSE OXIDATION BY RAT SPLEEN SLICES

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1. When washed spleen slices from fed rats are incubated with 3mM-[U-^{14}C]glucose, the rate of glucose utilization (46.2μmol/h per g dry wt.) is sufficient to account, theoretically, for 80% of the O_2 consumption. Measurement of net lactate production, however, and the fate of the radioactive carbon, indicates that the contribution of glucose to the respiratory fuel of the tissue is only 25–30%, whereas 60–70% of the glucose utilized is converted into lactate. At saturating glucose concentrations (above 5mM) its contribution to the respiratory fuel of the slice is increased to a maximum value of 34–39%. Only 2% of the glucose utilized is metabolized via the oxidative steps of the pentose phosphate pathway. 2. Starvation for 72h marginally increases both the rate of glucose utilization (by 21%) and its net contribution to the respiratory fuel (by 29%). 3. Insulin, glucagon, adrenaline and adenosine 3′:5′-cyclic monophosphate have no significant effect on either the rate of glucose utilization or on the pattern of radioactive isotope distribution. 4. The uptake of glucose is increased by only 20%, whereas the production of lactate doubles when slices are incubated under anaerobic conditions. 5. In assessing the suitability of spleen slices for metabolic studies, the only serious major perturbation, compared with the freeze-clamped organ, is an elevated mitochondrial [NAD^+]/[NADH] ratio (connected with increased endogenous NH_3 production) that is partially restored to normal values on incubation with glucose. 6. Equal proportions of erythrocytes and leucocytes are found in the washed spleen slice. Metabolic contributions of the constituent cell populations in the washed slice are calculated and it is concluded that lymphocytes account for the major part of the glycolytic metabolism (80–90%), whereas the contribution of erythrocytes is insignificant.

Although the immunological role played by lymphocytes and lymphoid tissues in the mammal is under intensive investigation at present, there is little detailed biochemical information available on the regulation of oxidative metabolism in intact lymphoid tissues. Indeed, no quantitative information exists on the normal respiratory fuel of the mammalian spleen (Krebs, 1972) and, by implication, no systematic study has been made of the factors that regulate the choice of fuel in this tissue. We have recently begun such a study in this laboratory and, in this paper, report experiments aimed at establishing the quantitative contribution made by [U-^{14}C]glucose to the oxidative metabolism of the normal rat spleen as an example of metabolic regulation in a secondary lymphoid tissue.

The limited information available on glucose oxidation by rat spleen slices indicates that a high proportion of the glucose utilized is converted preferentially into lactate (Dickens & Greville, 1933). This is in harmony with the view that lymphocytes, by analogy with the mouse spleen (Moore & Metcalf, 1971), constitute more than 90% of the total leucocyte content of the rat tissue. Human blood lymphocytes (Hedeskov & Esmann, 1966, 1967) and chicken leucocytes (Racker, 1965) both exhibit a similar pattern of glucose utilization, in that the major part of the glucose carbon taken up is incompletely oxidized to lactate. The results reported here for rat spleen confirm and extend the earlier studies and show that, although the total glucose taken up by spleen slices is sufficient to account quantitatively for 80–100% of the tissue respiration (if all of the glucose utilized were completely oxidized), approx. 60–70% is converted into lactate, and glucose oxidation accounts finally for only 25–30% of the O_2 consumption.

It is also shown that glucose metabolism by the tissue is insensitive to a number of hormones (including insulin) and that incubation in an anaerobic

environment fails to show a significant Pasteur effect.

Experimental

Incubation procedure

Spleen slices from at least six male Wistar rats (200–300g) were cut free-hand by the method of Deutsch (1935) and after being pooled were washed for 10min at 35°C, to remove endogenous metabolites, in phosphate-buffered saline prepared as described by Krebs & de Gasquet (1964), but modified to contain low Ca^{2+} and Mg^{2+} concentrations: 116ml of 0.154M-NaCl; 4ml of 0.154M-KCl; 3ml of 0.055M-$CaCl_2$; 1ml of 0.011M-$MgSO_4$; 1ml of 0.154M-K_2HPO_4; 6.5ml of 0.1M-sodium phosphate buffer, pH7.4. Slices (150–200mg, 4–6/flask) were taken at random from the pool and incubated in either Warburg or Erlenmeyer flasks at 38°C in 2.0ml of the same saline with 100% O_2 as the gas phase. Variation in the amount of tissue added per flask (in the range 50–300mg) caused no significant change, on a dry-weight basis, in any of the parameters measured.

The availability of O_2 and metabolites added to the incubated slices was not considered to be limiting. The mean thickness of the slices used in a number of experiments was 0.045±0.002cm. This is well below the maximum thickness of spleen slices (0.064cm) calculated to produce limiting diffusion of O_2 through the tissue (Umbreit *et al.*, 1964). In addition, no change in O_2 uptake was recorded when the shaking rate of the flasks was increased from 101 oscillations/min to 146 oscillations/min. All subsequent incubations were carried out at the higher shaking rate.

Incubations were terminated by the addition of 0.5M-HCl (0.25ml) from the side arms to liberate CO_2 and the flasks were shaken for a further 15min (until cessation of pressure changes). $HClO_4$ (0.25ml; 20%, w/v) was then added to the flasks, which were left on ice for a further 30min until the medium was prepared for analysis as described by Gevers & Krebs (1966). The respiratory CO_2 was collected and estimated manometrically as described by Krebs *et al.* (1966). The initial dry weights of the slices were obtained by dividing the individual wet weights, determined before incubation, by the wet-weight/dry-weight ratio obtained with identical unincubated slices that were subsequently dried for 16h at 125°C.

Reagents

[U-^{14}C]Glucose, [1-^{14}C]glucose and [6-^{14}C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Diazyme (amyloglucosidase) was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Freeze-clamping

Animals were stunned with a light blow on the head and killed by cervical dislocation. The spleens were removed rapidly after severing the blood supply and were crushed immediately between two aluminium plates pre-cooled to −180°C in liquid N_2 (Wollenberger *et al.*, 1960). The frozen tissue was weighed and immediately transferred to a porcelain mortar containing liquid N_2. $HClO_4$ (3ml; 12%, w/v) was then added, dropwise, to the mortar and the frozen material was ground to a powder and transferred quantitatively into an ice-cooled Teflon–glass tissue homogenizer (A. H. Thomas, Philadelphia, Pa., U.S.A.). After homogenization, the homogenate was centrifuged at 48000*g* for 15min to remove the protein pellet. The pellets were dried for 16h at 125°C and weighed to determine the dry weight of starting tissue.

The supernatants were adjusted to pH5.5 with K_2CO_3 (5M), and were centrifuged at 27000*g* for 10min to remove the precipitated $KClO_4$. The unstable intermediates were assayed immediately and the remaining solution was stored at −180°C. The results are expressed on a fresh-weight basis by using the dry weights of the individual samples and a conversion factor obtained by subjecting control spleens to an identical $HClO_4$ extraction step before drying.

Analytical methods

The metabolites were determined spectrophotometrically by the following enzymic methods: NH_3 by the method of Kirsten *et al.* (1963); aspartate by the method of Pfleiderer (1965); glutamate by the method of Bergmeyer & Bernt (1965*b*); glucose by the hexokinase method (Slein, 1965); glycogen by the method of Bartley & Dean (1968); L-lactate by the method of Hohorst (1965); ATP by the method of Adam (1965). Pyruvate was determined by using a fluorimetric adaptation of the method of Bücher *et al.* (1965); α-oxoglutarate by a fluorimetric adaptation of the method of Bergmeyer & Bernt (1965*a*), and acetoacetate and β-hydroxybutyrate by a fluorimetric adaptation of the methods of Williamson *et al.* (1962). It was found, after exhaustive testing, that the glucose oxidase method (Krebs *et al.*, 1963) could not be used for glucose determination in spleen tissue extracts, probably owing to the interaction of *o*-dianisidine with an unknown substance produced by the tissue.

Radiochemical methods

Separation of radioactive metabolites. [U-^{14}C]-Glucose, [^{14}C]lactate, [^{14}C]glutamate and [^{14}C]-aspartate present in the $HClO_4$ extract at the end of

the experiment were separated on a Dowex 1 column (acetate form) as described by Busch (1953). Nucleic acids and protein were extracted from the washed homogenized slices and separated as described by Munro *et al.* (1964), and the lipids were isolated as described by Folch *et al.* (1957). The radioactivity in the respiratory CO_2 was determined as given in Weidemann & Krebs (1969).

Determination of radioactivity in tissue glycogen. The radioactivity present in glycogen and glucose oligosaccharides was determined by a modification of the enzymic procedure described by Bartley & Dean (1968). After termination of slice incubations by addition of 0.5M-HCl (0.25ml), as described above, slices and medium were transferred quantitatively into an ice-cold glass–glass tissue grinder (Pyrex–Corning, New York, N.Y., U.S.A.) and the homogenate prepared was transferred quantitatively into a cooled graduated tube and made up to 4.5ml with aqueous washings from the tissue grinder.

The total radioactivity (in glucose, oligosaccharides and glycogen) was determined as [U-^{14}C]glucose by addition of sodium acetate buffer (1.0ml; 3M, pH4.4) and Diazyme (0.15ml; 10mg/ml) to 2.0ml of the homogenate, followed by incubation at 50°C for 30min (Bartley & Dean, 1968). The incubation was terminated by the addition of $HClO_4$ (0.5ml; 20%, w/v) and, after 30min extraction on ice, prepared for analysis as described above. Total [U-^{14}C]glucose was separated from other $HClO_4$-soluble compounds (Busch, 1953) on a Dowex 1 column (acetate form) and counted for radioactivity as described below.

The net radioactivity present in glycogen plus oligosaccharides was determined by subtracting the radioactivity present in the free unmetabolized [U-^{14}C]glucose remaining in the slice plus medium from this total. The unmetabolized [U-^{14}C]glucose radioactivity was recovered from a portion (2.0ml) of the homogenate treated as above, but substituting 0.15ml of water for the Diazyme suspension. A significant amount of soluble glycogen and oligosaccharide was also extracted from the tissue during this procedure. Since these compounds were eluted from the Dowex 1 column simultaneously with [U-^{14}C]glucose, the extent of the contamination by glycogen and oligosaccharide radioactivity was determined by separation on pre-coated silicic acid t.l.c. plates (ITLC-SA, Gelman, Calif., U.S.A.) eluted with chloroform–acetic acid–water (6:7:1, by vol.).

Radioactivity counting procedure. Radioactivity in the separated products was determined by liquid-scintillation counting in a Packard Tri-Carb liquid-scintillation spectrometer. Methoxyethanol–toluene (400–600ml, v/v) containing 6g of butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; Packard Instrument Co., La Grange, Ill., U.S.A.] was used as the scintillation medium.

Erythrocyte- and leucocyte-counting procedures

Cell suspensions from both washed and unwashed spleen slices of known wet weight were obtained by rubbing the tissues with forceps against a nylon mesh (500μm) partially immersed in NaCl (10ml; 0.9%, w/v) until the residual tissue was white in colour.

Total viable cells (erythrocytes plus leucocytes) as determined by the Trypan Blue-exclusion test (Black & Berenbaum, 1964) were counted in a haemocytometer and the proportions of erythrocytes and leucocytes were estimated by two independent methods. Method (i): slides were prepared of cell suspension stained with Wright's stain (Gurr, 1962) and the proportion of erythrocytes (brick-red, no nucleus) and leucocytes (nuclei stained blue) were counted in a population of at least 50 cells. Method (ii): cell suspensions were treated with leucocyte-counting fluid (aqueous solution of 0.1% acetic acid and 0.01% Methyl Violet, w/v) which, after 5min, lyses all erythrocytes, leaving the leucocytes intact. The leucocytes were counted subsequently in a haemocytometer. The erythrocytes were determined by subtracting the number of leucocytes present from the total number of cells extracted from the spleen slice. All results are expressed as no. of cells/g dry wt. of tissue.

Results and Discussion

Suitability of spleen slices for metabolic experiments

In this and subsequent papers, sliced rat spleen is used as a model to study quantitative changes in carbohydrate metabolism in rat lymphoid tissue. Implicit in the use of the slice is the assumption that the concentration of intermediates and cofactors reflect as nearly as possible the intracellular conditions in the tissue *in vivo*.

Adenine nucleotide ratios

Washed spleen slices appear to retain approx. 65% of their initial preincubation adenine nucleotide content even after 60min incubation, whether in the presence or absence of 3mM-glucose (Table 1). The slow, fairly linear, loss of nucleotides contrasts with the situation found in guinea-pig cerebral-cortex slices and rat liver slices where, for example, an extensive initial fall in adenine nucleotide content occurs during the first 5min of incubation (to 57 and 25% respectively of the initial values) and there is little further loss during the next 50–60min (Krebs, 1969; Rolleston & Newsholme, 1967). In liver slices only the nucleotides retained in the mitochondrial matrix space appear to be resistant to removal by washing with physiological saline (Krebs, 1969). In this respect spleen slices resemble more closely those

Table 1. *Variation of individual adenine nucleotide content during incubation of rat spleen slices*

Spleen slices incubated for the period shown were removed from the incubation medium, blotted to remove adherent medium and homogenized in cold $HClO_4$ (2%, w/v) to extract adenine nucleotides for assay. For other experimental details see the text.

Experimental procedure	Substrate added	Nucleotide concentrations (μmol/g fresh wt. of tissue)			Energy-charge ratio $\frac{1}{2}\left(\frac{2ATP+ADP}{ATP+ADP+AMP}\right)$
		ATP	ADP	AMP	
Freeze-clamped *in vivo*	None	2.62	0.71	0.09	0.87
Washed slices					
Unincubated	None	2.39	0.65	0.10	0.87
Incubated 20min	None	1.66	0.55	0.09	0.84
	Glucose (3mM)	1.73	0.56	0.09	0.85
Incubated 40min	None	1.64	0.55	0.10	0.84
	Glucose (3mM)	1.47	0.49	0.09	0.84
Incubated 60min	None	1.40	0.49	0.09	0.83
	Glucose (3mM)	1.42	0.47	0.09	0.84

prepared from rat kidney cortex, where the total nucleotide content falls by only 30% after 35min incubation with 2mM-glucose (Krebs, 1969; Underwood & Newsholme, 1967).

Additional relevant information may be derived from measurements of the relative amounts of the individual nucleotides in the slice during the time-course of an incubation (Table 1). In spleen slices, although the ATP and ADP values reached 54 and 66% respectively of the freeze-clamped value *in vivo* after 60min of incubation, the AMP content did not deviate from its original value *in situ* (100μM) and the energy-charge ratio remained high (0.84). Consistent with this finding, the linearity of the O_2 consumption, glucose utilization and lactate production of spleen slices during 90min of incubation (results not shown) suggests that there are no major quantitative or kinetic alterations in net carbohydrate metabolism that may be attributable to adenine nucleotide fluctuations.

Nicotinamide nucleotide ratios

The high activity of lactate dehydrogenase in the 50000*g* supernatant prepared from rat spleen homogenates (89.5±2.7μmol/min per g fresh wt.) allows determination of the cytoplasmic free [NAD^+]/[NADH] ratio from the lactate and pyruvate contents measured in the tissue plus medium (Table 2). It should be noted that the high lactate concentration found on incubation of the slices may represent, in part, a continuing loss of lactate from the slice until the extracellular concentration in the medium reaches or exceeds the approximate physiological value (about 1mM); for this reason, and because pyruvate concentrations undergo a similar percentage increase on incubation, the metabolite concentrations in the tissue plus medium have been used to calculate the [lactate]/[pyruvate] ratio. The cytoplasmic free [NAD^+]/[NADH] ratio estimated in incubated slices (Table 3) was essentially similar to the freeze-clamped value determined in the well-fed rat spleen, but tended to be slightly more decreased when the slices were incubated with 3mM-glucose. No significant differences were found in slices from starved spleens, in contrast with rat liver (Williamson *et al.*, 1967), although the reduction of the cytoplasmic couple by glucose was even more pronounced in this case.

The activities of the β-hydroxybutyrate dehydrogenase and glutamate dehydrogenase systems in rat spleen are 28 and 281±43mol of NADH formed/h per g fresh weight of tissue respectively (Williamson *et al.*, 1967). Both of these activities are low compared with rat liver, and since no specific statement can be made about the activities required to maintain equilibrium, we have compared both systems. Comparison of the results with those quoted for rat liver (Williamson *et al.*, 1967) show that the values obtained from the glutamate dehydrogenase system (3.3±0.07, five observations) are similar to liver, whereas those from the β-hydroxybutyrate system (60.9±32.4, five observations) are higher by one order of magnitude. This difference, and the extremely large variation in [NAD^+]/[NADH] ratios calculated from the β-hydroxybutyrate dehydrogenase system, suggests that the activity of β-hydroxybutyrate dehydrogenase is too low to maintain equilibrium in this tissue. Thus the [NAD^+]/[NADH] ratio of the mitochondrial matrix may be conveniently calculated from the glutamate dehydrogenase system.

In contrast with the cytoplasmic ratio, incubation of slices without added substrate gave rise to a much more oxidized [NAD^+]/[NADH] couple in the mitochondrial matrix compared with the freeze-clamped value. During incubation with glucose there

Table 2. *Concentrations of the substrates of NAD$^+$-linked dehydrogenase systems in spleen of well-fed and starved rats*

Freeze-clamping and slice incubations were carried out as described in the text. Incubations were terminated by the addition of $HClO_4$ (0.25ml; 20%, w/v), and after homogenization of medium plus slices, the $HClO_4$ extract was prepared for analysis as described by Gevers & Krebs (1966). The concentrations of the metabolites assayed in the medium plus slices are expressed as μmol/g fresh wt. of tissue (mean±S.E.M. with numbers of observations in parentheses). The values given for freeze-clamped tissue are for five independent experiments whereas numbers of observations for slice values refers to replicate experiments from a large pool (10 spleens).

Nutritional state	Tissue state	Metabolite concn. (μmol/g fresh wt.) [Lactate]	[Pyruvate]	[Glutamate]	[α-Oxoglutarate]	$[NH_4^+]$	[β-Hydroxybutyrate]	[Acetoacetate]	[Lactate]/[Pyruvate]	[Glutamate]/[Oxoglutarate]×$[NH_4^+]$	[β-Hydroxybutyrate]/[Acetoacetate]
Well-fed	Freeze-clamped (5)	0.89±0.07	0.081±0.006	3.78±0.74	0.0882±0.009	0.55±0.15	0.03±0.09	0.089±0.2	11.2±0.6	83.2±17	0.33±0.11
	Incubated slices, no added substrate (5)	2.15±0.35	0.21±0.035	2.02±0.9	0.022±0.002	4.87±0.13			10.7±2.1	18.7±1.3	
	Incubated slices + glucose (3mM) (6)	14.8±3.0	1.14±0.25	2.2±0.04	0.0196±0.0007	2.73±0.12			13.2±1.2	41.2±2.0	
Starved	Incubated slices, no added substrate (4)	2.4±0.16	0.27±0.05						9.3±2.1		
	Incubated slices + glucose (3mM) (7)	15.4±1.3	1.23±0.14						12.6±1.4		

was only a small decrease in the cytoplasmic ratio, whereas the extent of the decrease in the mitochondrial matrix was much greater and moved towards the value *in vivo*. The difference between the mitochondrial [NAD$^+$]/[NADH] ratio in the freeze-clamped (3.3±0.07) and incubated sliced tissue (13.9±0.9) can be readily understood from the 10-fold increase in NH_3 and twofold decrease in α-oxoglutarate (Table 2) which accompanied incubation (Lund *et al.*, 1970). The apparent deviation of the ratio from the freeze-clamped value is probably primarily due to the production of high concentrations of NH_3 and its possible uneven distribution between the intra- and extra-cellular compartments (Lund *et al.*, 1970), and this may be one of the more serious major perturbations caused by the slicing and oxygenation of the tissue.

Quantitative aspects of [U-^{14}C]glucose metabolism

Glucose concentration dependence. Glucose utilization increased with increasing initial glucose concentration until saturation was achieved at approx. 5mM (Table 4). There was, however, no significant change in the gas exchange (O_2 consumption or CO_2 production). Lactate and pyruvate production continued to increase even at saturating glucose concentrations, although the [lactate]/[pyruvate] ratio remained the same.

The percentage contribution made by glucose to the respiratory fuel can be calculated from these values on two different bases: method (i), by the net glucose uptake not accounted for by conversion into lactate plus pyruvate; and method (ii), by dilution of the specific radioactivity of the carbon atoms derived from [U-^{14}C]glucose recovered in the CO_2. The agreement between the two calculations is very good when the endogenous lactate production is subtracted in making the calculation by method (i) (Table 4), giving a value of 34–39% at saturating glucose concentrations as the likely maximum contribution of glucose oxidation to the respiratory fuel of spleen slices from the well-fed rat.

Net changes at fixed glucose concentration. Subsequent incubations using glucose as substrate were carried out at a glucose concentration of 3mM, near the freeze-clamped tissue concentration of 2mM and close to saturation. The results of such experiments for both well-fed and 72h-starved rats are shown in Table 5.

There was no significant difference in O_2 consumption by spleen slices when incubated with and without glucose in either dietary state. However, the respiratory quotient rose in the presence of glucose in both states, suggesting that carbohydrate oxidation had become quantitatively more important.

In the presence of substrate, 70–80% of the total

Table 3. *Calculation of [NAD$^+$]/[NADH] ratios in rat spleen from the concentration of the oxidants and reductants of the lactate dehydrogenase, glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase systems*

The free nicotinamide nucleotide ratios in cytoplasm, mitochondrial matrix and mitochondrial cristae space were calculated as described by Williamson *et al.* (1967). In each case the incubation pH was assumed to be 7.0. The results are given as means ± S.E.M.

Nutritional state	Tissue state	Lactate dehydrogenase system (cytoplasm)	Glutamate dehydrogenase system (mitochondrial matrix)	β-Hydroxybutyrate dehydrogenase system (mitochondrial cristae)
Well-fed	Freeze-clamped	818 ± 50	3.3 ± 0.07	60.9 ± 32.4
	Incubated slices, no added substrate	877 ± 160	13.9 ± 0.9	
	Incubated slices, + glucose (3 mM)	688 ± 59	6.29 ± 0.3	
Starved	Incubated slices, no added substrate	1003 ± 221		
	Incubated slices, + glucose (3 mM)	725 ± 75		

Table 4. *Effect of [U-^{14}C]glucose concentration on glucose utilization by rat spleen slices*

Spleen slices from well-fed animals were incubated for 60 min in the phosphate-buffered saline. For full experimental details see the text. The percentage contribution of glucose to the fuel of respiration was calculated as described below. The specific radioactivities of $^{14}CO_2$ and [U-^{14}C]glucose (used to calculated the percentage of the respiratory CO_2 derived from [U-^{14}C]glucose) are both expressed as c.p.m./μg-atom of C. The formation of metabolites is indicated by a + sign and disappearance by a − sign.

	Metabolic changes (μmol/h per g dry wt. of tissue)						
[U-^{14}C]Glucose concn. (mM) ...	0	0.4	0.7	1.3	2.5	4.9	9.7
O_2	−334	−337	−345	−326	−352	—	−311
CO_2	+285	+299	+275	+257	+278	+285	+246
Glucose	+0.8	−17.4	−25.8	−26.7	−47.4	−60.9	−61.1
Lactate	+13.4	+28.7	+36.4	+32.1	+64.0	+78.1	+89.7
Pyruvate	+0.96	+1.9	+2.7	+1.5	+4.6	+5.1	+5.8
[Lactate]/[pyruvate]	14.0	15.0	13.6	21.4	14.0	15.4	15.4
% contribution of glucose oxidation to O_2 consumption	—	15.7	22.4	30.6	33.8	—	38.5
$\frac{100 \times \text{sp. radioactivity of } ^{14}CO_2}{\text{Sp. radioactivity of [U-}^{14}\text{C]glucose}}$	—	12.1	17.7	23.4	28.5	30.1	35.6

Table 5. *Contribution of [U-^{14}C]glucose to the fuel of respiration of rat spleen slices from fed and starved rats*

Rat spleen slices were incubated for 60 min in the phosphate-buffered saline (2.0 ml). For full experimental details see the text and for the calculation of the contribution of glucose to the respiratory fuel on the basis of isotope dilution, see Table 4. The formation of metabolites is indicated by a + sign and disappearance by a − sign. The results are given as means ± S.E.M. for the numbers of experiments in parentheses. Values that were tested statistically for significant difference are indicated by $*P < 0.02$; †, not significant.

	Metabolic changes (μmol/h per g dry wt. of tissue)			
Substrate added ...	None		Glucose (3.0 mM)	
Nutritional state ...	Well-fed (9)	Starved (72 h) (2)	Well-fed (9)	Starved (72 h) (3)
O_2	−352 ± 25	−315	−342 ± 31	−328 ± 11
CO_2	+310 ± 33	+259	+328 ± 41	+292 ± 27
Respiratory quotient	0.88 ± 0.05	0.82	0.96 ± 0.05	0.89 ± 0.08
Glucose	+1.28 ± 0.26	—	−46.2 ± 2.7*	−56.0 ± 1.8*
Lactate	+9.9 ± 1.1	+12.6 ± 0.6	+75.9 ± 13.8	+81.2 ± 7·8
Pyruvate	+0.79 ± 0.22	+1.4 ± 0.3	+5.8 ± 1.6	+6.5 ± 0.97
[Lactate]/[Pyruvate]	12.7 ± 2.8	9.4 ± 2.1	13.4 ± 1.15	12.6 ± 1.4
Aspartate	+17.6 ± 2.8		+12.2 ± 1.0	
Glutamate	+9.7 ± 0.9		+10.7 ± 0.4	
% contribution of glucose oxidation to O_2 consumption	—	—	24.4 ± 3.8*	31.6 ± 5.7*
$\frac{100 \times \text{sp. radioactivity of } ^{14}CO_2}{\text{sp. radioactivity of [U-}^{14}\text{C]glucose}}$	—	—	21.9 ± 2.7†	23.6 ± 2.0†
% suppression of endogenous respiration by glucose	0	0	22.4 ± 9.8	32.3 ± 4.8

glucose utilized by spleen slices from well-fed rats (46.2±2.7 μmol/h per g dry wt. of tissue) was accounted for by the formation of lactate plus pyruvate. In slices from starved rats, in contrast with kidney cortex (Weidemann & Krebs, 1969; Underwood & Newsholme, 1967), heart and diaphragm muscle (Randle *et al.*, 1964), glucose utilization increased by 21%.

On the assumption that the glucose not recovered in lactate and pyruvate was completely oxidized, the contribution made by glucose to the respiratory fuel was 24.4 and 31.6% in the well-fed and starved animals respectively. The dilution of the initial specific radioactivity of the glucose carbon in the respiratory CO_2 supports this net calculation in the well-fed animal. The larger difference between the values based on these two calculations in the starved animal suggests that the extra glucose utilized is not oxidized but is converted into some other unidentified metabolite. In both starved and well-fed animals the contribution of endogenous substrates to the oxidative fuel was suppressed by only 20–30% and we can conclude from this that, even at near-saturating glucose concentration, endogenous substrates contribute more than 70% of the oxidative fuel.

Radioactive isotope distribution. A typical example of the complete radioactive isotope distribution after oxidation of 3.0mM-[U-^{14}C]glucose by slices from a well-fed rat is given in Table 6. In general, the major fate of glucose revealed here is consistent with the predictions made on the basis of the net changes. Some 96% of the starting radioactivity was recovered in all of the metabolites isolated, indicating that there are no other major metabolic products. Of the radioactivity recovered, 50% was found in the lactate pool, 28% in the respiratory CO_2 and 8.6% was shared between glutamate and aspartate.

Of the radioactivity present in the water-insoluble material of the homogenized slice, most (8.3% of total radioactivity disappearing) was recovered in glycogen and oligosaccharides and the remainder (4.1%) in protein, nucleic acids and lipids.

The specific radioactivities of glutamate, aspartate and CO_2 recovered at the end of the experiment are essentially similar, which suggests that the amino acids are labelled by 'isotope exchange' through the glutamate transaminase and aspartate transaminase half-reactions (Haslam & Krebs, 1963; Krebs *et al.*, 1966). Of the two methods previously used for calculating the contribution of glucose to the oxidative fuel, more careful examination of Tables 4 and 5 shows that the calculation based on isotope dilution of the radioactivity recovered in the respired CO_2 gives values consistently lower than those calculated from the net changes. If the radioactivity incorporated into glutamate and aspartate is taken into account in assessing the glucose carbon potentially available for CO_2 production in the well-fed animal, then the two methods of calculation give virtually identical values.

The specific radioactivity of the lactate recovered at the end of the incubation indicates considerable dilution (65%) of the specific radioactivity of the starting glucose carbon during its conversion into lactate. Thus lactate production from endogenous sources appears to continue unsuppressed during the oxidation of exogenous glucose. This observation justifies the need, mentioned previously, to subtract the lactate produced from endogenous sources from the lactate formed in the presence of added glucose when assessing the contribution of glucose to the respiratory fuel on the basis of net changes. An obvious source of unlabelled glycolytic carbon that might contribute to dilution of the lactate radioactivity is glycogen. The amount of glycogen in the slice before incubation was measured (8.5 μmol of glucose equivalents/g dry wt.) and calculation shows that the maximum dilution of the lactate carbon that could occur from this source is only 10%. Further, measurement of the glycogen remaining in the slice at the end of a 60min incubation showed no significant

Table 6. *Radioactive isotope distribution after oxidation of [U-^{14}C]glucose by spleen slices from the well-fed rat*

Slices were incubated for 90min. All other conditions were as described in Table 5.

Radioactivity recovered in	10^{-2} × Radioactivity recovered (c.p.m.)	% of recovered radioactivity (not including glucose)	10^{-4} × Sp. radioactivity (c.p.m./μg-atom of C)
Glucose (initial)	104063		26.48
Glucose (final)	46407		27.14
CO_2	15358	28.6	5.53
Lactate	27142	50.5	17.37
Glutamate	2588	4.8	8.63
Aspartate	2054	3.8	6.58
Glycogen+oligosaccharide	4439	8.3	19.47
Lipid	500	0.9	
Nucleic acid	362	0.7	
Protein	1346	2.6	
Total radioactivity recovered	100184		

net gain or loss from the pool. The source of the remaining unlabelled lactate remains unclear at this time.

Effect of anaerobiosis on glucose utilization

The incubation of spleen slices under anaerobic conditions increased the utilization of glucose by only 20% (Table 7). The lactate production was doubled in the presence of glucose and increased nearly sixfold when no substrate was added. The slight stimulation of glucose uptake was unexpected, since a more pronounced Pasteur effect has been demonstrated in the intact organ after 10min of ischaemia (Suter, 1973). The weak Pasteur effect observed with spleen slices is in direct contrast with findings in anaerobically perfused rat heart and liver (Morgan *et al.*, 1959; Woods & Krebs, 1971), anaerobically incubated rat diaphragm muscle (Randle & Smith, 1958) and kidney cortex, and cerebral-cortex slices incubated with cyanide (Rolleston & Newsholme, 1967). The anaerobic rate of lactate production by spleen slices when supplied with 3mM-glucose (145μmol/h per g dry wt.) was very similar to the rate of lactate production by the intact organ between 5 and 10min of ischaemia (154μmol/h per g dry wt.) and probably represents the maximum glycolytic capacity of this tissue (Suter, 1973).

The production of lactate from slices incubated anaerobically without added substrate was large (50.8μmol/h per g dry wt.) but much less than that found by Dickens & Greville (1933) (134μmol/h per g dry wt.). The total glycogen in washed spleen slices just before incubation could only account for 17μmol of lactate, assuming quantitative conversion. The source of the remaining 33mol of lactate formed is not definitely known. Although there is sufficient free alanine present in the tissue before incubation (18μmol/g dry wt.) to provide, through transamination, about one-half of the lactate unaccounted for, in liver at least, lactate and alanine both rise during anoxia (Brosnan *et al.*, 1970). The [lactate]/[glucose] ratio found in anaerobically incubated slices with added glucose was 2.66, which suggests that 70% of the lactate production from endogenous sources continued even in the presence of added glucose.

Effect of hormones and cyclic AMP on the oxidation of [U-^{14}C]glucose in incubated slices

One of the main objectives of the present work is to define conditions that provide the maximum capacity of the tissue for the uptake and oxidation of glucose. In other tissues, including cardiac and skeletal muscle, diaphragm and adipose tissue, the addition of insulin greatly increases the rate of glucose uptake (Williamson & Krebs, 1961; Ruderman *et al.*, 1971; Vallence-Owen & Hurlock, 1954; Denton *et al.*, 1968). Incubation of spleen slices with insulin (10nM or 2.9×10^{-3} i.u./flask) caused no significant change in either glucose uptake or its complete oxidation to CO_2. Similarly, glucagon (2.5μM) and adrenaline (10μM) did not cause any significant alteration in the overall metabolic pattern. Added cyclic AMP (1mM) did not cause any major changes in glycolysis or respiration except for a raised [lactate]/[pyruvate] ratio and a reversal of the suppression of NH_3 production by glucose. Although it is attractive to suggest that the latter finding may be the result of increased protein breakdown stimulated by cyclic

Table 7. *Effect of anaerobiosis on the metabolism of glucose* (3mM) *by rat spleen slices*

Slices were incubated as described in the text, except for anaerobic flasks, which were gassed with dry N_2 for 15s and contained convenient-sized sticks of yellow phosphorus in the inner well instead of 2M-KOH. Results are the means of duplicate incubations.

	Metabolic changes (μmol/h per g dry wt.)			
Atmosphere ...	Aerobic		Anaerobic	
Substrates added ...	None	[U-^{14}C]Glucose (3mM)	None	[U-^{14}C]Glucose (3mM)
O_2	−331	−330	—	—
CO_2	+317	+296	—	—
Respiratory quotient	0.96	0.90	—	—
Glucose	+0.8	−45.5	+1.4	−54.6
Lactate	+8.8	+67.8	+50.8	+145
Pyruvate	+0.95	+4.57	+0.18	+0.24
[Lactate]/[pyruvate]	9.3	14.85	281	627
$\frac{100\times\text{sp. radioactivity of } ^{14}CO_2}{\text{sp. radioactivity [U-}^{14}C_2\text{]glucose}}$	—	22.7		
% Contribution of glucose to the O_2 consumption	—	25.0		
[Lactate]/[glucose]	—	1.49	—	2.66
NH_3	38.0	26.0	26.9	24.2

AMP, it may be more simply explained by conversion of cyclic AMP into AMP by phosphodiesterase and the subsequent deamination of AMP by adenylate deaminase, which is known to be highly active in spleen (Setlow *et al.*, 1966; Brady & O'Donovan, 1965).

Contribution of the pentose pathway

In lymphoid tissue the pentose pathway is reported to have significant activity in human blood polymorphonuclear leucocytes during phagocytosis (Selvaraj & Bhat, 1972; DeChatelet *et al.*, 1972) and in peritoneal macrophages from the guinea pig (Oren *et al.*, 1963; Karnovsky & Wallach, 1963), whereas human blood lymphocytes have been shown to metabolize only 2% of their glucose consumption via this pathway *in vitro* (Hedeskov, 1968).

The contribution of the oxidative route of the pentose pathway to the glucose metabolism of the normal rat spleen was estimated from the $^{14}CO_2$ yield from specifically labelled glucose by three different methods: that of Beck (1958) and those of Katz *et al.* (1966). These are summarized in Table 8 and calculations are presented based on the radioactivity recovered in respiratory CO_2 (Table 8). Methods (*b*) and (*c*) indicate that the metabolism of glucose carbon by the oxidative segment of the pentose pathway was very low, accounting for only 2% of the catabolized glucose, whereas method (*a*) give a higher value of 6.1%. However, method (*a*) is probably less accurate, since it does not take into account the effect of recycling fructose 6-phosphate to glucose 6-phosphate; these hexose phosphates are almost certainly in equilibrium in the rat spleen, as the activity of phosphoglucose isomerase (back reaction) is 31.5 ± 1.3 μmol/g fresh wt. of tissue.

The contribution of the pentose pathway may also be estimated by measurement of the incorporation of specifically labelled glucose into a triose phosphate derivative. Method (*d*) considers incorporation of glucose radioactivity into lactate, as described by Katz *et al.* (1966). This method gives a value of 2.4%, in good agreement with methods (*b*) and (*c*).

Cellular composition of spleen slices

The spleen contains an extremely heterogeneous cell population which, from its deep-red colour, clearly includes a large number of erythrocytes. The cells of the spleen are associated with one or other of its major functions: the storage and filtration of blood and immunological reactions.

Table 8. *Calculation of the pentose pathway activity during metabolism of glucose by rat spleen slices*

Spleen slices were incubated with 3.0mM-[1-^{14}C]glucose and 3.0mM-[6-^{14}C]glucose in separate flasks (5×10^6 c.p.m./flask). For full experimental conditions see the text. Results are the means of triplicate incubations with each specifically labelled glucose. PC, percentage contribution of the pentose pathway to glucose metabolism. References for methods are: method (*a*), Beck (1958); methods (*b*), (*c*), (*d*), Katz *et al.* (1966).

Method (*a*) $PC = \dfrac{[1\text{-}^{14}CO_2] - [6\text{-}^{14}CO_2] \times 100}{\text{Glucose utilized}}$

Method (*b*) $PC = \dfrac{S \times 100}{3 - 2S}$ $\quad S = (G\text{-}1\text{-}CO_2) - (G\text{-}6\text{-}CO_2\alpha)$;

$$\alpha = \frac{^{14}\text{C from [1-}^{14}\text{C]glucose into lactate}}{^{14}\text{C from [6-}^{14}\text{C]glucose into lactate}}$$

Method (*c*) $PC = \dfrac{S \times 100}{3 - 2S}$ $\quad$ where $S = \dfrac{(G\text{-}1\text{-}CO_2) - (G\text{-}6\text{-}CO_2)}{1 - (G\text{-}6\text{-}CO_2)}$

and $G\text{-}CO_2$ is the specific yield of CO_2

Method (*d*) $PC = \dfrac{(1 - \alpha) \times 100}{1 + 2\alpha}$

Glucose labelled in position	Radioactivity recovered in $^{14}CO_2$ (c.p.m./flask)	Glucose utilized/flask (c.p.m.)	Specific yield	$\frac{1\text{-}^{14}CO_2}{6\text{-}^{14}CO_2}$	Radioactivity in lactate (c.p.m./flask)	$\frac{[1\text{-}^{14}\text{C]Glucose into lactate}}{[6\text{-}^{14}\text{C]Glucose into lactate}}$
1-C	195011	1415752	13.8		772973	
				1.52		0.93
6-C	121524	1331878	9.1		828948	

Calculated percentage contribution of pentose phosphate pathway by method:

(*a*)	(*b*)	(*c*)	(*d*)
6.1	1.83	1.78	2.37

Table 9 shows the results of an experiment designed to estimate the relative proportions of erythrocytes and leucocytes (all other nucleated cells) in the spleen slice before and after washing in a phosphate-buffered saline. The number of cells lost from the slice during washing is small, being only 10% of the total, most of which are erythrocytes. Also after washing, the cells remaining in the slice consist of approximately equal numbers of erythrocytes and leucocytes.

Composition of leucocytes

Although no information is available giving the type and distribution of the nucleated cells found in the rat spleen, this has been described for the mouse spleen (Moore & Metcalf, 1971) and it has been suggested (J. L. Gowans, personal communication) that essentially the same cellular composition might apply to the rat. The analysis quoted by Moore & Metcalf (1971) is qualified with the caution that differential counts on cells teased out of the spleen may not be truly representative of the entire leucocyte population, since cells more adherent to the structural framework of the spleen may not be readily released. With this qualification, typical data from an analysis of the cells present in an adult mouse spleen cell suspension are: lymphocytes 90.4%, nucleated erythroid cells 2.2%, polymorphonuclear leucocytes 5.2%, reticulum cells and macrophages 1.5% and plasma cells 0.7%.

It follows that lymphocytes and erythrocytes are nearly equal in number and represent by far the greatest proportion of the spleen-cell population.

Metabolic contribution of cell populations

Table 10 shows a comparison of glycolytic and respiratory rates for isolated cell suspensions of the main cell constituents of the rat spleen. Most of the glycolytic activity is found in lymphocytes, whereas erythrocytes make an insignificant contribution. The contribution of polymorphonuclear leucocytes is significant in spite of their small representation, as they have an extremely high aerobic glycolysis (Hedeskov & Esmann, 1966).

The 'reconstructed spleen slice' represents the sum of the average of the individual metabolic contributions made by each of the main constituent cell populations. In terms of glycolytic rates there is good agreement with those observed in the spleen slice, but there is a large discrepancy in the case of the observed respiratory rate, which is approximately six times

Table 9. *Proportion of erythrocytes and leucocytes in the rat spleen slice*

Cell suspensions from spleen slices were prepared and counted as described in the text. Slices were washed in phosphate buffered saline as described in the text. Unwashed slices were 'teased' immediately after slicing. Results are expressed as number of cells/g dry wt. of sliced tissue.

	Leucocytes	Erythrocytes	Total cells
Unwashed slices	$4.73\times10^9\pm1.76\times10^9$	$7.9\times10^9\pm1.96\times10^9$	$1.26\times10^{10}\pm0.08\times10^{10}$
Washed slices	$5.6\times10^9\pm1.3\times10^9$	$6.6\times10^9\pm1.15\times10^9$	$1.14\times10^{10}\pm0.17\times10^{10}$

Table 10. *Comparison of respiratory and glycolytic rates observed individually in various cell constituents of the rat spleen*

Rates are expressed as μmol/h for the total no. of cells expected/g dry wt. of spleen slice. The proportion of nucleated cell types found in the rat spleen is taken from data quoted for the mouse spleen (Moore & Metcalf, 1971). The 'reconstructed spleen slice' represents the respiratory and glycolytic rates expected from a direct summation of the contributions made by the constituent cell types.

Cell type	Reference	Cell source	Glucose concn. (mM)	O_2 consumption	Glucose utilization	Lactate production
Lymphocytes	Hedeskov & Esmann (1966)	Human blood	16.7	−59	−31	+48
	Rauch *et al.* (1961)	Human blood	5.0	−77.5	−35.4	+87.5
	Packman (1967)	Horse blood	5.6	−31.0	−54	+69.0
Polymorpho-nuclear leucocytes	Rauch *et al.* (1961)	Human blood	5.0	−2.5	−5.9	+10.0
	Hedeskov & Esmann (1966)	Human blood	16.7	−3.0	−11.5	+17.0
Erythrocytes	Hedeskov & Esmann (1967)	Human blood	16.7	0	−0.7	+1.7
Spleen-cell suspension	Culvenor (1972)	Wistar rat	5.0	—	—	+54
'Reconstructed spleen slice'				−58.6	−49.5	+83.4
Spleen slice		Wistar rat	3.0	-342 ± 31	-46.2 ± 2.7	$+75.9\pm13.8$

higher than that anticipated on the basis of calculation. It is possible, as suggested by Moore & Metcalf (1971), that there may be a cell population (possibly 'fixed' macrophages) remaining firmly attached to the spleen's travecular framework after preparation of cell suspensions. Macrophages are known to have a high respiratory rate (Oren *et al.*, 1963; Dannenberg *et al.*, 1963) and may utilize endogenous lipids as their principal fuel. Indeed, there is some evidence in favour of this idea (Suter, 1973). When the residual pulp remaining after cell-suspension preparation is incubated under the conditions described in the text, it is found to have a respiratory rate (on a dry-wt. basis) three times higher than that of the cell suspension derived from it.

The results reported and discussed above do suggest strongly that the glycolytic metabolism of the rat spleen slice is essentially that of the lymphocyte population, whereas most of the respiratory activity may be due to the oxidation of endogenous substrates (chiefly triacylglycerol) by tissue macrophages.

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