Regulation of Carbohydrate Metabolism in Lymphoid Tissue

NATURE OF THE ENDOGENOUS SUBSTRATES AND THEIR CONTRIBUTION TO THE RESPIRATORY FUEL OF THE SLICED RAT SPLEEN *IN VITRO*

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1. Tissue glycogen contributes, maximally, only 10% of the respiratory fuel of the rat spleen slice in the absence of an added carbon source, and makes no significant contribution when glucose (3 mM) is added. 2. The reserves of fatty acid in the form of triglyceride (35.5 μ mol of fatty acid/g dry wt. of tissue) fall by approx. 25% after incubation of spleen slices with or without added glucose for 2 h, and, on this basis, account for 32% of the oxidative fuel. 3. In contrast, the total oxidative contribution of fatty acid reserves to the respiratory fuel, determined on the basis of inhibition of respiration by 2-bromostearate, is 42–52%. This range includes tissue from both starved and well-fed animals and is not significantly altered by the presence of added glucose (3 mM). 4. Large quantities of NH_3 (31–35 μ mol/h per g dry wt. of tissue) are produced by spleen slices incubated in the absence of added substrates, and this value is suppressed by approx. 50% on incubation with glucose (3 mM). Adenine nucleotide breakdown can account for only 17% of the total ammonia produced. 5. Individual free amino acid concentrations in spleen were determined, both *in vivo* and in slices before and after 60 min of incubation. Although the total free amino acid pool size increases by 45% during incubation, owing to protein breakdown, the tissue concentrations of aspartate, glutamate, glutamine and alanine do not increase. It is suggested that these amino acids are oxidized in a net sense to CO_2 and water with the liberation of free NH_3 via transamination reactions, glutaminase, the purine nucleotide cycle and the tricarboxylic acid cycle. 6. It is concluded that the normal endogenous metabolism of sliced rat spleen (43–52% due to lipids, 30% due to amino acids and 10% due to glycogen) is modified by added glucose only to the extent that glycogen oxidation and 50% of the contribution made by amino acids are suppressed; endogenous lipid metabolism is unaffected.

Previous work from this laboratory (Suter & Weidemann, 1975) has shown that $[\text{U-}^{14}\text{C}]$ glucose oxidation accounts, quantitatively, for less than 40% of the oxidative metabolism of washed rat spleen slices and does not stimulate the respiratory rate of the tissue. The major portion of the glucose taken up is converted into lactate, suggesting that the oxidation of pyruvate does not compete effectively with endogenous substrates capable of generating alternate sources of acetyl-CoA.

In the present paper we have attempted to quantify the relative contributions made by tissue glycogen, lipids and amino acids to the oxygen consumption of the spleen slice. The influence of added glucose on the contributions made by each of these endogenous fuels to the total tissue respiration is also considered.

It has long been known that many tissues

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produce large quantities of ammonia during incubation of sliced preparations (Dickens & Greville, 1933; Weil-Malherbe & Green, 1955), but the source of this ammonia has never been clearly defined. Ammonia production by spleen slices, which is only partially suppressed by added glucose, is associated with an apparent rise in the mitochondrial $[\text{NAD}^+]/[\text{NADH}]$ ratio, when this value is calculated on the assumption that glutamate dehydrogenase catalyses a near-equilibrium reaction (Suter & Weidemann, 1975). In the present paper we suggest and present some evidence to support the view that ammonia is produced in spleen slices partly through glutaminase activity and partly through the operation of the purine nucleotide cycle. Amino acids produced by proteolysis during incubation of tissue slices provide the aspartate that is required for maintenance of the cycle and, in doing so, their carbon skeletons are ultimately oxidized to CO_2 and water through the tricarboxylic acid cycle.

Experimental

Materials

Preparation of 2-bromostearate-bovine serum albumin complex. Bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) was freed of contaminating long-chain fatty acids by the method of Chen (1967). A weighed quantity (43.6mg) of potassium 2-bromostearate (K & K Laboratories, Plainview, NY, U.S.A.) was suspended in 0.25ml of hot distilled water, and 15ml of calcium-free double-strength phosphate-buffered saline (pH 7.4; Suter & Weidemann, 1975) containing 8% defatted bovine serum albumin was added, with constant stirring, to give a bromostearate concentration of 8.0mM. The mixture was sonicated at maximum power for a total of 30min at 37°C (100 W Ultrasonic Disintegrator; MSE, London S.W.1, U.K.) until only a fine suspension of 2-bromostearate remained, and was then incubated at 37°C for 1h, with constant agitation, until the suspension cleared. CaCl_2 (0.1ml of 375mM) was added, and the solution was diluted twofold to give a final 2-bromostearate concentration of 4.0mM. In experiments where this complex was used to inhibit respiration, control incubations containing no bromostearate were carried out in the presence of 4% defatted bovine serum albumin alone.

Chemicals. Other chemicals used were of the highest purity available commercially and were not further purified.

Methods

Incubation procedure. Unless otherwise indicated, slices were prepared and incubations carried out at 38°C as described by Suter & Weidemann (1975).

Freeze-clamping. Whole spleens were freeze-clamped *in situ* as described previously (Suter & Weidemann, 1975).

Analytical methods

Enzymic assay of metabolites. The following metabolites were determined spectrophotometrically by enzymic methods: glucose, L-lactate and pyruvate as previously described (Suter & Weidemann, 1975); glycogen as glucose by the method of Bartley & Dean (1968); ammonia by the method of Kirsten *et al.* (1963); and glutamine by the method of Lund (1974). In the latter method hydroxylamine (2mM) was included in the incubation medium during glutamine hydrolysis to inhibit glutamate decarboxylase activity associated with the glutaminase preparation used (Sigma Chemical Co., grade II, lot 124C-6800).

Triglyceride determination. The triglyceride content of the freeze-clamped whole spleen and pooled spleen slices was determined as glyceride glycerol after alkaline hydrolysis of a Folch extract (Folch *et al.*, 1957) by using a Biochemica Test Combination Kit

supplied by Boehringer Mannheim G.m.b.H., Mannheim, Germany (catalogue reference 15989 TNAA).

Protein determination. The total protein content of the intact spleen and spleen slices was measured on homogenates of the tissue by the biuret method (Layne, 1957).

Amino acid analysis

Preparation of tissue extract. The tissue (freeze-clamped whole spleen or pooled slices) was extracted at 0°C with HClO_4 for 30min as described previously (Suter & Weidemann, 1975). The HClO_4 extract was separated from the protein pellet after centrifugation at 48000g for 15min, neutralized to pH 4–5 with K_2CO_3 (5M) and made up to 10ml with water. Precipitated KClO_4 was removed by centrifugation at 20000g for 10min. The amino acids in the extract were separated from glycolytic and tricarboxylic acid-cycle intermediates by passing a sample of the extract (4ml) through a Dowex 50 W (H^+ form) ion-exchange column (20cm $\times$ 1.5cm) (Sigma). The contaminating carboxylic acids were eluted with water until the pH of the eluate was neutral. The adhering amino acids were subsequently eluted with NH_3 (2.5M), and the eluate (20–30ml) was collected as the solvent front, indicated by a brown ring, neared the end of the column. By using a Buchi rotary evaporator, this eluate was evaporated and redissolved in water a number of times, until the pH of the distillate indicated no further release of ammonia.

Automatic amino acid analysis. The dried sample was dissolved in 1.0ml of Technicon analyser starting buffer [0.05M-trisodium citrate buffer, adjusted to pH 2.75 with 6M-HCl plus 10% methanol, 0.5% thiodiglycol and 0.4% Brij-35 (polyoxyethylene cetyl ether)] containing 12% sucrose. Samples (0.2ml) were injected on the column of a Technicon autoanalyser (Technicon Corp., Tarrytown, NY, U.S.A.) packed with Chromobeads (C-2 resin) and eluted with a continuous pH gradient (pH 2.75–6.10). The amino acids in the eluate were detected by scanning at 570nm and 440nm, and the peaks obtained were integrated with a Technicon integrator. Lysine, histidine and arginine, which were determined in extracts prepared from freeze-clamped spleens, could not be determined with sufficient accuracy on slice extracts owing to a rising baseline at the end of the run, so these three amino acids have been omitted from the total free amino acid pool values reported in Table 4.

Results and Discussion

Glycogen

The content of glycogen found in the freeze-clamped spleen *in vivo* is $14.57 \pm 1.2 \mu\text{mol}$ of glucose

Table 1. Concentrations of potential endogenous fuels in the rat spleen *in vivo* and in spleen slices

Glycogen, protein and triglycerides were measured in the intact rat spleen and in slices (just before incubation) as described in the text. The glycogen concentration is expressed as μmol of glucose equivalents/g dry wt. of tissue. On the assumption that most of the neutral glyceride is triglyceride, the values given are expressed as μmol of fatty acid equivalents/g dry wt., where there are three fatty acyl groups per glycerol unit. The protein concentration is given as mg/g of dry tissue. Also, on the assumption that the average molecular weight of the amino acid residues in this protein is approx. 110, the protein concentration is also expressed as μmol of amino acid equivalents (McGilvery, 1970). Results are $\pm$ S.E.M., for the numbers of observations given in parentheses. The fresh wt./dry wt. ratio was 4.30 for freeze-clamped spleens and 4.75 for freshly prepared slices.

Fuel available	<i>In vivo</i>	Slices
Glycogen	14.57 $\pm$ 1.2 (6)	8.6 (7.9, 9.2) (2)
Protein	800 $\pm$ 58 (5)	855 $\pm$ 133 (5)
Protein (amino acid equivalents)	7260	7770
Triglyceride	37.8 $\pm$ 10.5 (6)	35.5 $\pm$ 11.2 (5)

equivalents/g dry wt. (Table 1). In slices, the tissue content of glycogen fell to approx. 50% of that *in vivo* (8.6 μmol /g dry wt.) just before incubation (Table 1). The rate of glycogen utilization in the absence of added glucose (5.6 μmol of glucose equivalents/h per g dry wt. of tissue) could account, maximally, for only 34 μmol of oxygen consumed, or approx. 10% of the oxidative fuel. In the presence of glucose there was no net utilization of glycogen during 60 min of incubation, and therefore under these conditions it made no significant contribution to the respiratory fuel.

Fatty acids (from endogenous triglyceride)

Net disappearance. The reserves of fatty acid available in the form of triglyceride were measured *in vivo* and in the slice (Table 1) and were found to be 37.8 and 35.5 μmol of fatty acid/g dry wt. of tissue respectively, indicating that there was little or no loss of triglyceride during preparation of the slices. Assuming that the average carbon-chain length of the fatty acids derived from neutral triglyceride is 18, the complete oxidation of 1 mol of fatty acid requires approx. 25 mol of oxygen (Weidemann & Krebs, 1969). Thus the amount of fatty acid available in the slices just before incubation (35.5 μmol) is sufficient to maintain the respiratory rate (352 μmol of O_2 /h per g dry wt.) for about 2½ h, provided that no other substrates are utilized. Clearly, triglyceride may be an important source of endogenous respiratory fuel. The concentration of triglyceride in freshly prepared slices decreased by 25% during 2 h of

incubation whether added glucose was present or not, and this deficit could account for approx. 32% of the oxidative fuel. However, because of the extremely large S.E.M. observed in the triglyceride concentrations measured before incubation (Table 1), a specific inhibitor of fatty acid oxidation (2-bromostearic acid) was used as an alternative method of estimating the contribution of endogenous fatty acid oxidation to the respiratory fuel.

Estimation of endogenous triglyceride fatty acid contribution to the respiratory fuel by using 2-bromostearate. 2-Bromostearate is a powerful inhibitor of palmitoyl-CoA-carnitine acyltransferase, an enzyme which plays a specific role in the oxidation of fatty acids (Tubbs & Garland, 1968). Perfusion of hearts from alloxan-diabetic rats with 2-bromostearate has been shown to restore impaired glucose utilization by selectively inhibiting the oxidation of fatty acids (Randle, 1969). Bringolf *et al.* (1972) also found increased pyruvate and glucose oxidation in diaphragms from rats maintained on a high-fat diet after incubation with 2-bromostearate. This was shown to be due to the selective inhibition of diaphragm glyceride oxidation.

The inhibition of respiration in spleen slices from well-fed and starved rats (72 h) by 2-bromostearate is shown in Table 2. Slices were preincubated with 2-bromostearate for 40 min before addition of glucose and initiation of gas-exchange measurement, as it was found that the inhibition of oxygen consumption was essentially linear after this time. On the assumption that the inhibited respiration in the absence of added glucose is directly equivalent to the inhibition of fatty acid oxidation, then in both dietary states and with no added substrate present, endogenous fatty acid oxidation contributes approx. 43–52% of the oxidative fuel.

The inhibition of respiration observed with 3.0 mM-glucose present is more difficult to interpret as, contrary to expectation, glucose uptake and the oxidation of glucose carbon to CO_2 (as judged from the total radioactivity recovered in the respiratory CO_2) fell by 20–30% on addition of 2-bromostearate in both dietary states. The inhibition of respiration must therefore include, in addition to that due to the inhibition of fatty acid oxidation, a portion equivalent to the suppression of labelled CO_2 production from [$\text{U-}^{14}\text{C}$]glucose shown in Table 2. When this is taken into account, the portion of the oxygen consumption specifically due to fatty acid oxidation in the presence of 3.0 mM-glucose is approx. 42% in the well-fed state and 48% after 72 h of starvation.

The inhibition of glucose uptake in the presence of 2-bromostearate may be readily understood from the fall in the cytoplasmic free $[\text{NAD}^+]/[\text{NADH}]$ ratio calculated from the $[\text{lactate}]/[\text{pyruvate}]$ ratio observed in the medium at the end of the incubation

Table 2. *Inhibition of oxygen consumption and [U-¹⁴C]glucose metabolism by 2-bromostearate in spleen slices from 72h-starved and well-fed rats*

All flasks were preincubated for 40 min with 2-bromostearate but without added glucose. After addition of 3.0 mM-[U-¹⁴C]glucose (specific radioactivity 49870 d.p.m./μg-atom of C) to the relevant flasks, gas exchange was monitored over a further 60 min of incubation. The formation of metabolites is indicated by a + sign and disappearance by a - sign. The cytoplasmic free [NAD⁺]/[NADH] ratio was calculated from the [lactate]/[pyruvate] ratio as described by Williamson *et al.* (1967). The results are given for three experiments ± s.e.m. For further experimental details see the text.

Substrate added ...	(μmol/h per g dry wt. of tissue)									
	None					[U- ¹⁴ C]glucose (3.0 mM)				
	None		2-Bromostearate (4 mM)			None		2-Bromostearate (4 mM)		
Inhibitor added ...										
Nutritional state ...	Well-fed	Starved	Well-fed	Starved		Well-fed	Starved	Well-fed	Starved	
O ₂	-336 ± 11	-352 ± 18	-191 ± 22	-168 ± 14		-336 ± 4	-326 ± 26	-174 ± 6	-150 ± 18	
CO ₂	+305 ± 13	+354 ± 23	+151 ± 19	+155 ± 19		+317 ± 3	+319 ± 28	+128 ± 8	+138 ± 35	
Inhibition of O ₂ consumption (%)			43	52				48	54	
Glucose	+5.3 ± 0.7	+7.7 ± 1.3	+23.2 ± 2.4	+29.0 ± 0.8		-61.7 ± 2.9	-54.3 ± 1.2	-49.1 ± 4.2	-38.6 ± 2.2	
Lactate	+0.52 ± 0.07	+0.46 ± 0.11	+0.78 ± 0.04	+0.47 ± 0.07		+62.1 ± 10.6	+77.2 ± 8.0	+93.2 ± 3.7	+102 ± 6.2	
Pyruvate	10.1	16.7	29.6	61.7		+4.1 ± 0.3	+5.47 ± 0.09	+0.77 ± 0.2	+0.90 ± 0.07	
Cytoplasmic [NAD ⁺]/[NADH] (calculated)	867	535	303	147		15.1	14.1	121	114	
Radioactivity recovered in CO ₂ (d.p.m./flask)						594	644	83	79	
Specific radioactivity of CO ₂ (d.p.m./μg-atom of C)						171 000	155 060	121 330	107 870	
Sp. radioactivity of ¹⁴ CO ₂ × 100						11 400	10 700	15 260	17 220	
Sp. radioactivity of [U- ¹⁴ C]glucose						22.9	21.5	30.6	34.5	

Table 3. Contribution of endogenous amino acids to the oxidative fuel of the rat spleen slice calculated from ammonia production during incubation

Slices were incubated for 60min at 38°C as described in the text. The tissue was extracted for analysis as described previously (Suter & Weidemann, 1975). In calculating the percentage contribution of amino acids to the oxidative fuel it was assumed that on average there is one amino group per amino acid and that the average number of carbon atoms in each amino acid is 4.25. It has also been assumed that 5.5 μmol of NH₄⁺/h per g dry wt. of slice is derived from breakdown of adenine nucleotides. Results are means ± s.e.m. for the numbers of observations given in parentheses.

Substrate added ...	Unincubated slices (μmol/g dry wt. of tissue)		Incubated slices (μmol/g dry wt. of tissue)			
	None		None		Glucose (3 mm)	
	Well-fed	Starved (72h)	Well-fed	Starved (72h)	Well-fed	Starved (72h)
Ammonia	8.4 ± 0.7 (4)	—	39.7 ± 3.7 (7)	43.5 (2)	27.1 ± 2.8 (7)	28.3 ± 2.0 (4)
Net ammonia production	—	—	+31.3	+35.1	+18.7	+19.9
Total adenine nucleotides	14.9 (2)	—	9.4 (2)	—	9.4 (2)	—
Approximate contribution of amino acids to O ₂ consumption (%)			29.6	40.0	15.6	19.0

Fig. 1. Effect of glucose concentration on the production of ammonia by rat spleen slices

The incubations were performed at 38°C as described in the text. The results given are the means of duplicate incubations.

(Table 2). This would lower both the availability of NAD⁺ for glycolysis and the steady-state concentration of cytoplasmic pyruvate available for transport into the mitochondrion. The reason for the highly reduced state of the cytoplasmic free nicotinamide nucleotide ratio in the presence of 2-bromostearate, which has not been observed in other tissues, is not known, but may be connected with an impaired ability of the mitochondrial dicarboxylate carrier to transport reducing equivalents, as malate, into the mitochondrial matrix for oxidation. Thus, although the percentage contribution of glucose

oxidation to the remaining oxygen consumption undergoes a modest increase when fatty acid oxidation is inhibited with 2-bromostearate (as evidenced by the rise in specific radioactivity of the respired CO₂ shown in Table 2), this effect is ultimately limited by secondary perturbations of the oxidation-reduction state of the cytoplasmic nicotinamide nucleotides, which restrict glycolytic flux.

Amino acids

Ammonia production. Many tissues, including spleen, produce large quantities of ammonia when sliced and incubated (Dickens & Greville, 1933; Weil-Malherbe & Green, 1955; Suter & Weidemann, 1975). Glucose addition usually suppresses ammonia production (Dickens & Greville, 1933; Weil-Malherbe & Green, 1955), but this effect is only partial in spleen slices (Table 3). At 20mm-glucose, which is nearly twice the concentration used to produce a total inhibition of ammonia production in brain slices (Weil-Malherbe & Green, 1955), the inhibition in spleen slices was only 66% (Fig. 1).

There are several alternative deaminating enzyme systems that may be associated with the ammonia production observed in tissue slices: glutaminase, glutamate dehydrogenase, amine oxidase, adenylate deaminase and adenosine deaminase. Weil-Malherbe & Green (1955) concluded that ammonia production in brain was not derived from any of these systems in a net sense.

Spleen is known to have extremely active adenylate deaminase and adenosine deaminase (the activities at 37°C are 2.7 μmol of NH₄⁺/min per g fresh wt. and 17.6 μmol of adenosine/min per g fresh wt. respectively; Setlow *et al.*, 1966; Brady & O'Donovan, 1965). During 60min of incubation there was a significant decline in the concentration of

Table 4. Comparison of amino acid concentration in the freeze-clamped rat spleen *in vivo* and in rat spleen slices before and after incubation

The incubations were performed for 60 min as described in the Experimental section. Other experimental details are given in the text. Results are presented as means $\pm$ S.E.M., with numbers of observations given in parentheses.

Amino acid	Content ($\mu\text{mol/g}$ dry wt.)			
	<i>In vivo</i>	Unincubated slices	Incubated slices	
Substrate added ...	(5)	None (5)	None (3)	Glucose (3 mM) (3)
Aspartate	9.1 $\pm$ 0.62	13.10 $\pm$ 0.51	13.65 $\pm$ 0.78	9.43 $\pm$ 0.16
Glutamate	13.88 $\pm$ 0.95	7.85 $\pm$ 0.61	5.03 $\pm$ 1.13	7.73 $\pm$ 0.86
Glutamine	5.99 $\pm$ 0.4	3.17 $\pm$ 0.44	0.77 $\pm$ 0.17	0.71 $\pm$ 0.11
Alanine	5.52 $\pm$ 0.4	10.27 $\pm$ 0.97	11.27 $\pm$ 1.16	11.51 $\pm$ 1.52
Glycine	7.9 $\pm$ 0.9	11.56 $\pm$ 1.53	17.02 $\pm$ 2.5	16.19 $\pm$ 2.2
Serine	3.4 $\pm$ 0.34	5.24 $\pm$ 0.69	10.42 $\pm$ 0.4	11.40 $\pm$ 0.62
Threonine	6.3 $\pm$ 0.6	2.24 $\pm$ 0.4	5.74 $\pm$ 0.5	6.06 $\pm$ 1.54
Proline	—	1.65 $\pm$ 0.09	3.63 $\pm$ 2.4	3.58 $\pm$ 1.4
Valine	0.41 $\pm$ 0.2	1.40 $\pm$ 0.12	4.92 $\pm$ 0.93	4.52 $\pm$ 0.1
Methionine	—	0.54	1.15 $\pm$ 0.49	0.63 $\pm$ 0.22
Isoleucine	0.47 $\pm$ 0.25	0.60 $\pm$ 0.07	2.79 $\pm$ 0.49	2.59 $\pm$ 0.26
Leucine	0.86 $\pm$ 0.30	1.96 $\pm$ 0.10	6.59 $\pm$ 1.2	6.27 $\pm$ 0.28
Tyrosine	0.95 $\pm$ 0.56	0.98 $\pm$ 0.82	2.50 $\pm$ 0.18	2.28 $\pm$ 0.26
Phenylalanine	0.73 $\pm$ 0.41	0.73 $\pm$ 0.11	3.33 $\pm$ 0.4	2.94 $\pm$ 0.27
Total pool size	55.51 $\pm$ 3.6	61.29 $\pm$ 1.7	88.8 $\pm$ 11.8	85.8 $\pm$ 4.9
Lysine	2.4 $\pm$ 0.73	—	—	—
Histidine	0.95 $\pm$ 0.65	—	—	—
Arginine	0.82 $\pm$ 0.22	—	—	—

total adenine nucleotides, which could liberate a maximum of 5.5 μmol of ammonia/h per g dry wt. of tissue. However, this represents only a small part of the total ammonia production (18%).

Spleen has also been shown to possess many of the key enzymes involved in the transamination and deamination of some amino acids, e.g. glutaminase (22 $\mu\text{mol/min}$ per g fresh wt.), glutamate dehydrogenase (281 $\mu\text{mol/min}$ per g fresh wt.) and glutamate-oxaloacetate transaminase (1950 $\mu\text{mol/min}$ per g fresh wt.) (Krebs, 1972; Williamson *et al.*, 1967; Greenstein & Leuthardt, 1948). It seems reasonable to suggest that the deamination of amino acids is a major source of the ammonia released during incubation.

In an attempt to test this hypothesis, free amino acids were measured both *in vivo* and in spleen slices before and after incubation (Table 4). The total pool size was slightly larger (11%) in the unincubated slices than *in vivo*, suggesting that some proteolysis takes place during the preparation of the slices. The total pool size in slices, however, increased by 45% after 60 min of incubation irrespective of whether glucose was present in the incubation medium or not. Lysosomal particles in spleen leucocytes are known to be particularly vulnerable to mechanical injury of the type that may occur during the slicing, washing and incubation of the tissue (Bowers *et al.*, 1967).

Evidently proteinases released from these particles are activated during the preparation of the tissue and catalyse significant proteolysis during the subsequent period of incubation at 38°C.

Most of the individual amino acid concentrations increased 2–4-fold during the incubation period, except for aspartate, glutamate, glutamine, alanine and, possibly, glycine, which either maintained their preincubation concentrations or, for glutamine, declined significantly. Because the enzymes associated with the catabolism of at least four of these amino acids are known to be present in spleen (see above) it is proposed that their tissue concentrations do not increase during incubation because the rate of amino acid release by proteolysis is counteracted by their immediate removal for oxidative deamination.

There are three reactions by which net production of ammonia from aspartate, glutamine, glutamate and alanine might be achieved (Scheme 1). The glutaminase reaction is the one most likely to be the source of net ammonia from the glutamine released by proteolysis. This is confirmed by the rapid disappearance of glutamine from the tissue during 60 min of incubation. However, the glutamate released from this reaction, from proteolysis and through transamination of alanine plus α -oxoglutarate or aspartate plus α -oxoglutarate, is unlikely to release ammonia through the glutamate dehydro-

1. The glutaminase reaction:

2. The glutamate dehydrogenase reaction:

3. The purine nucleotide cycle:

Scheme 1. Reactions by which net production of ammonia from amino acids might be achieved in spleen slices

genase reaction. This is because the equilibrium position of the reaction, under physiological conditions, strongly favours the reductive amination of α -oxoglutarate. The free energy change (ΔE) at pH 7.0 with NAD^+ is 28.03 kJ (Lowenstein, 1972). Indeed, using isolated rat liver cells, Mendes-Mouřao *et al.* (1975) have demonstrated that glutamate dehydrogenase does not produce net quantities of NH_3 at a sufficient rate to account for urea synthesis.

On the other hand, the overall free energy change (ΔE) for the following set of reactions involved in the purine nucleotide cycle (Lowenstein, 1972), which could well be applied to the spleen slice situation, is -4.18 kJ, favouring deamination:

Scheme 1, considered as a whole, would allow the complete oxidation of these four amino acids to CO_2 and water through the tricarboxylic acid cycle: glutamine and glutamate would maintain tricarboxylic acid-cycle intermediates at the level of α -oxoglutarate, aspartate would ensure a ready supply of oxaloacetate, and alanine would supply acetyl-CoA. Since only the carbon atoms of the acetyl moiety are oxidized to CO_2 , and the remaining four carbon atoms act as a carrier, the acetyl-CoA concentration would be sustained by maintenance of alanine concentrations through the operation of the two transaminase enzyme systems (glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase).

Net reaction:

Reaction (1) may also be omitted, in which case the reaction is:

Reaction (1) may also be preceded by:

in which case the net reaction would be:

Table 5. *Effect of added amino acids on oxygen consumption and production of ammonia by rat spleen slices incubated in the presence and absence of added glucose*

Slices were incubated for 120 min at 38°C as described in the text. Results are expressed as μmol of oxygen consumed or ammonia produced/h per g dry wt. $\pm$ S.E.M. where three incubations were carried out or as averages of duplicates where only two incubations were performed.

Amino acid added ... None		Aspartate (5mm)	Glutamate (5mm)	Glutamine (5mm)	Alanine (5mm)	Glycine (5mm)	
Glucose added	Parameter measured						
None	O ₂ consumption	301 ± 34	306 ± 35	308 ± 28	318 ± 43	288 ± 39	330 ± 50
	Ammonia	30.9 ± 5.6	41.7 ± 8.4	30.5 ± 6.8	115.9 ± 13.1	36.5 ± 6.8	33.3 ± 6.6
0.5mm-glucose	O ₂ consumption	326	334	350	325	319	363
	Ammonia	20.1	26.1	14.7	89.8	22.8	19.1
5.0mm-glucose	O ₂ consumption	351	377	319	351	339	351
	Ammonia	21.3	26.7	16.4	55.1	22.3	22.0

The partial inhibition of ammonia production by added glucose (see Table 3 and Fig. 1) can be explained in terms of an increase in the steady-state concentration of tissue pyruvate produced by glycolysis so that the equilibrium of the glutamate-pyruvate transaminase reaction shifts towards alanine formation. In this way the acetyl-CoA derived from amino acids via alanine is considerably diluted by acetyl-CoA derived from glucose carbon. There was no increase in the total pool size of the endogenous free amino acids (Table 4) after incubation with glucose, however, which suggests that glucose metabolism may slightly inhibit the breakdown of protein in addition to suppressing amino acid oxidation. The small but consistent decrease in the concentrations of most of the free amino acids that are not rapidly oxidized is in agreement with this prediction.

Amino acids added to slices exogenously, except for glutamine, failed to increase the basal ammonia production significantly, and did not oppose its suppression by glucose oxidation (Table 5). Permeability barriers at the level of the plasma membrane may restrict entry of these amino acids into the cells or, alternatively, a key step in the reaction sequence (1)–(10) may be already saturated with substrate at the concentrations of glutamate, aspartate and alanine present in the intracellular free amino acid pool. In contrast, glutamine was readily deaminated by slices in the absence of glucose, and the production of ammonia from this source was suppressed progressively (up to 50%) as the concentration of glucose was raised to 5.0 mm. The rapid disappearance of endogenous glutamine from the free amino acid pool during the preparation and incubation of slices (Table 4) is a further reflexion of the significant glutaminase activity present in this tissue. No explanation can be offered at present for the partial inhibition of this reaction by glucose or one of its metabolites.

Contribution of endogenous amino acids to oxidative fuel. In calculating the percentage contribution of endogenous amino acids to the oxidative fuel it is assumed that on average there is one amino group per amino acid and that the average number of carbon atoms in each amino group oxidized (aspartate, glutamine, glutamate, alanine) is 4.25. It has also been assumed that 5.5 μmol of NH_4^+ /h per g dry wt. of slice is derived from the breakdown of adenine nucleotides. On this basis, amino acids appear to make a significant contribution to the respiratory fuel in the absence of added substrate in the fed animal (29.6%) and account for 40% of the endogenous substrate in the starved animal. Incubation with glucose (3 mm), however, suppresses the oxidation of amino acids in both dietary states by approx. 50%. It should be noted that the values given in Table 3 slightly overestimate the contribution of endogenous amino acids to the respiratory fuel to the extent that the glutaminase reaction continues to contribute significant net ammonia production at the low steady-state concentration of glutamine measured in the incubated slice (Table 4).

Contribution of endogenous fuels. Fatty acids derived from endogenous triglyceride provide most of the respiratory fuel *in vitro* in slices from the starved and well-fed animal (Table 6). Amino acids derived from protein breakdown may account for most of the remaining oxidative fuel in slices prepared from animals on both dietary regimes, although their contribution is apparently decreased by 50% in the presence of glucose. Table 6 shows that in both dietary states tested, and in the presence and absence of glucose, the percentage of the oxidative fuel unaccounted for is only 8–20%. This supports the assumptions used in estimating the oxidation of fatty acid reserves by 2-bromostearate inhibition and in determining the contribution made by endogenously formed amino acids to the oxidative fuel of the rat spleen slice.

Table 6. Contributions of endogenous substrates to the oxidative fuel of rat spleen slices in the presence and absence of added glucose (3 mM)

Percentage contributions of glucose to the respiratory fuel are taken from Suter & Weidemann (1975). Calculations were carried out as described in the text.

Nutritional state ...	Percentage contribution to respiratory fuel			
	Starved		Well-fed	
	Glucose		Glucose	
Additions ...	None	(3 mM)	None	(3 mM)
Substrates				
Triglyceride (endogenous)	52	48	43	42
Amino acid (endogenous protein)	40	19	30	16
Glycogen (endogenous)	—	—	10	0
Glucose (3 mM)(exogenous)		24		22
Percentage of oxygen consumption unaccounted for	8	9	17	20

It is unlikely that this situation is truly representative of the tissue *in vivo*. Although added amino acids do not cause an increase in oxygen consumption (Table 5), as they do in the liver, the rather large proportion of the existing oxygen consumption that has been attributed to endogenous amino acid catabolism in the present study must be due, at least in part, to an increased proteolysis, which is an artifact of slicing and oxygenation of the tissue. This may represent one of the major difficulties encountered in extrapolating data obtained from preparations of spleen and other lymphoid tissues *in vitro*, where mechanical disruption of the tissue is involved, to the situation *in vivo*. In addition, externally added substrates, such as glucose, normally gain access to the tissue via an intact blood supply. The present results on the modification of the endogenous metabolic pattern by added glucose should therefore be regarded with some caution, since the relative and absolute rates of diffusion of substrates into the tissue may not be the same as those encountered *in vivo*.

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