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Energy Metabolism in *Leishmania*

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Alanine plays a key role in the response of promastigotes to osmotic stress and to hypoxia. It is rapidly released in response to hypo-osmolality, is consumed from its large intracellular pool under iso-osmotic conditions even in the presence of glucose, and is synthesized under hyper-osmotic conditions even in the absence of glucose. Its rate of oxidation, in the presence or absence of any of ten other amino acids tested, is strongly inhibited by hyperosmolality. Glucose oxidation is also inhibited by hyperosmolality, but to a lesser extent than that of alanine, and is inhibited by alanine, glutamate, and aspartate. Hyperosmolality also inhibits the incorporation of label from [2-^{14}C]acetate into the putative storage carbohydrate, mannan, which occurs via the glyoxylate bypass and the as yet unexplored "mannoneogenic" pathway. The rates of glycolysis and of oxidation of several amino acids decrease with increasing culture age, but the capacity to oxidize fatty acids increases, and in cells from 3-day stationary phase cultures hyperosmolality enhances rather than inhibits alanine oxidation.

KEY WORDS: *Leishmania*; osmotic effects; alanine; mannan; glucose.

1. INTRODUCTION

Several reviews of the organization and control of intermediary metabolism of the *Leishmania* and related trypanosomes have been published recently (Glew *et al.*, 1988; Cazzulo, 1992; Blum, 1993*a*,*b*). In this paper I shall therefore focus primarily on several aspects of metabolism and bioenergetics that have not been addressed in the above-mentioned reviews, such as the metabolic interactions between substrates and the bioenergetic implications of the presence of the glyoxylate cycle and a storage carbohydrate.

2. GLUCOSE CATABOLISM

Figure 1 diagrams the probable configuration of the pathways of glucose and fatty acid utilization and of the metabolism of four amino acids—alanine, glutamate, proline, and aspartate—that are metabolized via the Krebs cycle. The major products formed by *L. braziliensis*, *L. major*, and *L. donovani* incubated with glucose as sole exogenous carbon source are (in addition to CO_2) acetate, succinate, pyruvate, and a small amount of D-lactate (see Blum, 1993*a,b*, for details). For *L. (mexicana) pifanoi*, however, the major products in addition to CO_2 are succinate, malate, acetate, and alanine (Rainey and MacKenzie, 1991). For *L. major* and probably for other species as well, in the absence of both O_2 and CO_2, glucose consumption practically ceases (a complete "reverse" Pasteur effect), indicating that CO_2 is required for the reoxidation of the NADH formed in the glycosomes during anaerobic glycolysis (Darling *et al.*, 1989b). In Fig. 1, the only pathway by which this can occur is via the return of phosphoenolpyruvate (PEP)[2], formed in the cytosol by the terminal steps of the glycolytic pathway, to the glycosomes, where phosphoenol carboxykinase (PEPCK) can form OAA from PEP in the presence of CO_2, and the OAA can be reduced to malate (Opperdoes, 1987; Cazzulo, 1992). In *L. donovani*, *L. major*, and *L. braziliensis* virtually all of the malate presumably

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[2] Metabolite abbreviations are given in the legend to Fig. 1.

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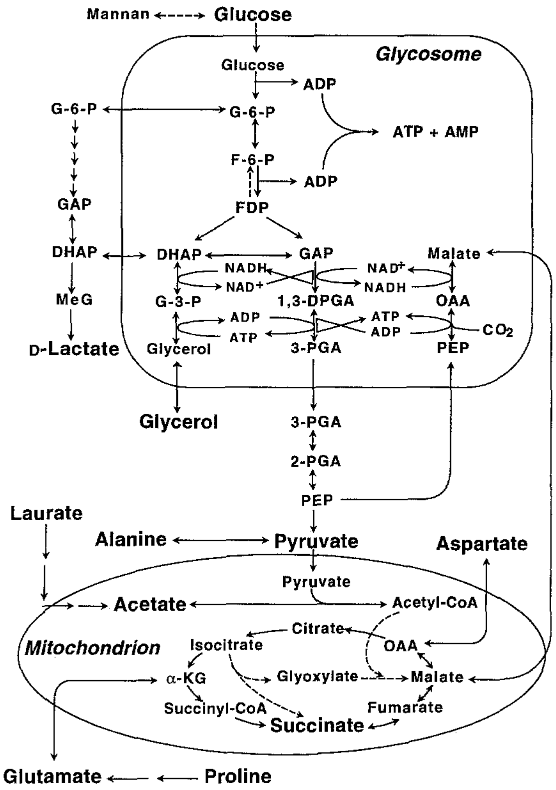

Fig. 1. Schematic diagram of the pathways of glucose and fatty acid metabolism in *L. major* and *L. donovani* and of the metabolism of alanine, glutamate, aspartate, and proline (modified from Blum, 1993a). The glyoxylate bypass is shown in the mitochondrion, although the localization of the isocitrate lyase and malate synthase activities have not yet been determined. For convenience, the β-oxidation pathway is shown as occurring in the mitochondrion, although several enzymes of the β-oxidation pathway also occur in the glycosomal fraction (Coombs *et al.*, 1982; Hart and Opperdoes, 1984; Opperdoes, 1990). The localization of fructose-1,6-bisphosphatase and of the mannan biosynthetic and degradative pathways are shown by dashed arrows in the glycosome and in the cytoplasm, respectively; uncertainties concerning their localization are discussed in the text. The localization of alanine, aspartate, and glutamate transaminases is also discussed in the text. Abbreviations used are: G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; FDP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; MeG, methylglyoxal; G-3-P, glycerol 3-phosphate; 1,3-DPGA, 1,3 bisphosphoglycerate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; 2-KG, 2-ketoglutarate.

returns to the mitochondrion, where it is converted to fumarate by the action of fumarase and then to succinate via fumarate reductase (Mracek *et al.*, 1991). The major difference between *L. m. pifanoi* and the other three species of *Leishmania* so far examined is therefore likely to be in the relative amounts of these enzymes. It should also be emphasized that both pyruvate carboxylase and malic enzyme, not shown in Fig. 1, are present in *Leishmania*, and may participate in CO_2 fixation and carbon balance, although PEPCK appears to play the major role, as discussed in detail by Cazzulo (1992).

Although very little D-lactate, pyruvate, or glycerol are formed by promastigotes incubated with glucose under aerobic conditions, appreciable amounts are formed when the pO_2 drops to 6% (Keegan and Blum, 1990). This indicates the presence of a low-affinity O_2 receptor, the nature of which is presently unknown. D-Lactate and glycerol formation presumably serve as overflow pathways under conditions where the rate of consumption of glucose exceeds the requirement for ATP production, as discussed in more detail elsewhere (Blum, 1993*a*). This overflow function is not likely, however, to explain why acute hyperosmotic stress under fully aerobic conditions causes an increased production of D-lactate by *L. donovani* promastigotes (Walsh and Blum, 1992). Unlike *L. major*, *L. donovani* appears not to have any methylglyoxal synthase (Ghoshal *et al.*, 1989). Van der Jagt *et al.* (1990) have suggested that the formation of D-lactate by *Plasmodium falciparum* may be a defense mechanism against the toxic effects of methylglyoxal formed nonenzymatically within infected erythrocytes, and a detoxifying function for the methylglyoxal pathway in *L. donovani*, *L. major*, and *L. braziliensis* has also been suggested (Darling and Blum, 1988), though this would seem unnecessary given the presence of glyoxylases I and II in these promastigotes. If one consequence of exposure to acute hyperosmolality is a decrease in cell water content and hence an increase in the concentration of intracellular metabolites, including intraglycosomal DHAP and GAP, then one might expect the rate of nonenzymatic formation of methylglyoxal to increase, which would account for the observed increase in D-lactate formation by *L. donovani* in response to acute hyperosmotic stress. Thus D-lactate formation in those species that do form D-lactate may serve not only as an overflow pathway under hypoxic conditions but also as a detoxifying pathway, as discussed in detail by Richard (1993).

In addition to its interest as a primary source of fuel and carbon for promastigotes, glucose is also of interest for its osmotic effects on these cells. When glucose or 2-deoxyglucose (2-DG) is added to *L. major* promastigotes suspended in Hanks' balanced salt solution, which is iso-osmotic to the growth medium, a slow (~ 20 min) change in cell shape occurs (Darling and Blum, 1990). This shape change is reversible (also ~ 20 min) upon removal of the glucose, is prevented by the simultaneous addition of mannitol (i.e., hyperosmolality), and is mimicked by the addition of H_2O (i.e., hypo-osmolality). These observations suggest that the accumulation of phosphorylated products of glucose metabolism, or, in the case of 2-DG, of 2-DG-6P (Schaefer *et al.*, 1974), causes an influx of H_2O, thus triggering the shape change. Support for this interpretation comes from studies on the kinetics of [U-^{14}C]glucose utilization in *T. brucei* (Visser *et al.*, 1981). These studies demonstrated the existence of two pools of glycolytic compounds, one of which—presumably the glycosomal pool—equilibrated in about 15 sec, while the other and much larger pool—presumably cytosolic—was not fully equilibrated after 10 minutes.

3. AMINO ACID METABOLISM

3.1. Alanine

Alanine, the largest single component of the intracellular pool of free amino acids in both promastigotes and amastigotes (Simon *et al.*, 1985; Mallinson and Coombs, 1989), plays a key role in the cell physiology of *Leishmania.* It is released rapidly in response to acute hypo-osmotic stress (Darling *et al.*, 1990), and synthesized—even in the absence of an exogenous carbon source—in response to acute hyperosmotic stress (Burrows and Blum, 1991). Its intracellular concentration also varies in response to hypoxia. Under aerobic conditions alanine is synthesized by *L. m. pifanoi* incubated with glucose as sole exogenous carbon source (Rainey and MacKenzie, 1991), but it is consumed (from the endogenous pool) by *L. major* (Keegan and Blum, 1990). Under hypoxic conditions, however, it is produced by both species. The source of the amino groups is likely to be via protein degradation, since *L. tropica* promastigotes have a labile class of proteins which are degraded more rapidly when the nutrients in the medium are exhausted or when energy metabolism is inhibited (Simon and Mukkada, 1983). In *L. major* promastigotes that were pre-labeled with [1-^{14}C]leucine and then washed and resuspended in buffer for 1 h under hypo-, iso-, or hyper-osmotic conditions, little proteolysis occurred, so it is not yet certain that the rise in alanine concentration in response to hyperosmolality results from proteolysis (Burrows and Blum, 1991). It should be noted that *T. cruzi* takes up and stores protein in organelles called reservosomes, and these proteins are degraded under nutritional stress (see Urbino, 1993). A comparative study of the presence and utilization of carbohydrate, lipid, and protein reserves in different species of Trypanosomatidae would be of considerable interest.

T. cruzi epimastigotes and *C. fasciculata* choanomastigotes consume glucose preferentially when both glucose and amino acids are present (Cazzulo *et al.*, 1985). In all species of *Leishmania* so far examined, however, amino acids from the medium are consumed in preference to glucose during exponential growth, with glucose consumption increasing during stationary phase (Krassner and Florey, 1971; Mukkada *et al.*, 1974; Marr and Berens, 1977). In view of the key role of alanine in the response of promastigotes to changes in osmolality and pO_2, it was of interest to examine the effects of glucose and of a number of amino acids on the rate of alanine oxidation by promastigotes suspended in buffer, especially since earlier studies had shown complex interactions between glucose and proline (Krassner and Florey, 1977).

Under iso-osmotic conditions, neither threonine, which is a preferred source of 2-carbon units for lipid synthesis in *T. brucei* (Klein and Linstead, 1976), nor histidine affected the rate of [U-^{14}C]alanine oxidation by *L. donovani* promastigotes (Table I). The presence of leucine, serine, or valine caused a small inhibition in the rate of alanine oxidation, but proline, glutamate, and aspartate appreciably increased the rate of $^{14}CO_2$ formation. Since as shown schematically in Fig. 1, proline is converted to glutamate and both glutamate and aspartate enter the Krebs cycle (as 2-KG and OAA, respectively), these data suggest that alanine oxidation by washed promastigotes is limited by the availability of OAA to condense with the acetyl CoA generated via the pyruvate dehydrogenase complex after transamination of the alanine.

In Fig. 1, alanine transamination is shown for convenience as occurring only in the cytoplasm. NMR studies on glucose utilization by *T. cruzi*, however, have demonstrated the presence of two pools of alanine (Frydman *et al.*, 1990), and a similar conclusion is compatible with the results of an NMR study on *L. braziliensis* (Darling *et al.*, 1989a). Presumably, most of the alanine is in the cytosol, from where it can be released into the medium in less than 1 min in response to acute hypo-osmotic stress (Darling *et al.*, 1990). The most likely location of the small second pool is within the mitochondrion, consistent with the presence of alanine transaminase in the mitochondrial fractions of *L. mexicana* (Mottram and Coombs,

Table I. Effect of Amino Acids and of Glucose on the Rate of Oxidation of Alanine under Iso-osmotic and Hyperosmotic Conditions

		Rate of [U-^{14}C]alanine oxidation (nmol h^{-1} (mg protein)$^{-1}$)		
Experiment number	Additions	305 mOsm kg^{-1}	610 mOsm kg^{-1}	% Inhibition
I	—	198 ± 14	$66.0 \pm 4.9^{***}$	67
($n = 4$)	Arginine	139 ± 6^c	$40.1 \pm 5.7^{***c}$	71
	Leucine	178 ± 12^a	$54.2 \pm 7.4^{***}$	70
	Serine	182 ± 15^a	$70.6 \pm 9.5^{***}$	61
II	—	146 ± 3^a	$42.0 \pm 8.9^{**}$	71
($n = 4$)	Proline	188 ± 20	$80.2 \pm 18.6^{***b}$	57
	Aspartate	200 ± 49^b	$56.6 \pm 6.8^{**b}$	72
III	—	160 ± 17	$50.5 \pm 10.9^{***}$	68
($n = 4$)	Lysine	123 ± 19^c	$36.2 \pm 5.0^{**a}$	71
	Threonine	145 ± 16	$50.2 \pm 12.0^{**}$	65
	Valine	141 ± 12^b	$33.5 = 7.8^{***b}$	76
IV	—	131 ± 29	38.3 ± 3.2^a	71
($n = 3$)	Histidine	124 ± 21	$28.0 \pm 4.5^{**b}$	77
	Glutamate	230 ± 17^c	$66.5 \pm 7.8^{***b}$	71
				69 ± 5 ($n = 14$)
V	—	137 ± 23	$48.2 \pm 8.9^{**}$	65
($n = 4$)	Glucose	187 ± 46^a	$155 \pm 44^{**b}$	17

$^{*,**,***}p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, for hyperosmotic vs. iso-osmotic conditions.

$^{a,b,c}p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, for the presence of an amino acid (or glucose) vs. its absence at a given osmolality.

L. Donovani promastigotes from late log phase cultures were collected, washed, and resuspended in Hanks balanced salt solution (HBSS$^-$) and incubated for 1 h at 26°C in the presence of 3 mM [U-^{14}C]alanine and 3 mM of the indicated unlabeled amino acids of 8 mM glucose. The % inhibition caused by the addition of 305 mM mannitol (i.e., at 610 mOsm kg^{-1}) is shown in the right-hand column.

1985), of *T. cruzi* (Duschak and Cazzulo, 1991), and of *T. brucei* (Opperdoes, personal communication). Glutamate dehydrogenase is also present in the mitochondrial (and cytosolic) fractions of *L. mexicana* and *T. cruzi*, and aspartate transaminase is present in the mitochondria of *T. brucei* (Opperdoes and Cottem, 1982). The dual localization of these enzymes (not shown in Fig. 1), is of interest since, as described in detail elsewhere (Blum, 1993b) both genistein, a protein-tyrosine kinase inhibitor, and staurosporine, an inhibitor of protein kinase C and of other protein kinases including tyrosine kinases, inhibit the oxidation of ^{14}C-labeled alanine, aspartate, glutamate (and of proline, which is oxidized to glutamate) but not that of glucose or laurate during a 1 h exposure of *L. donovani* promastigotes to these drugs. It is therefore likely that these protein kinase inhibitors interfere with the uptake and/or transamination/deamination of these amino acids. If the relevant transaminases are present in both the cytosolic and mitochondrial compartments, as appears to be the case, the possibility must be considered that these protein kinase inhibitors interfere with the uptake of alanine, glutamate, and aspartate into the mitochondrion and/or their subsequent transamination/deamination within that compartment instead of or in addition to interfering with uptake via the cell membrane and transamination within the cytosol. Studies on the effects of staurosporine and genistein on the amino acid uptake and transamination/deamination system of the cell membrane and of isolated mitochondria will be required to clarify these questions.

The data in Table I show that alanine oxidation, in the presence or absence of any of the 10 other amino acids examined, is inhibited about 69% by an increase is osmolality from 305 to 610 mOsm/kg. In the presence of glucose, however, hyperosmolality causes a much smaller (17%) inhibition of alanine oxidation. Presumably, the increase in intracellular concentration of glycolytic intermediates discussed above acts to counteract the osmotic effect of the externally added mannitol, thus minimizing cell water loss and the consequent inhibition of alanine oxidation.

3.2. Leucine

Steiger and Meshnik (1977) observed that glutamate, proline, and leucine were the three amino acids utilized most rapidly from the growth medium by both *L. braziliensis* and *L. donovani.* Leucine is catabolized to acetyl CoA and acetoacetate. The fate of the acetoacetate is uncertain; some or all of it may be converted to acetyl CoA (Blum, 1991). The rate of $^{14}CO_2$ formation from [U-^{14}C]leucine by *L. donovani* promastigotes from late log phase cultures was inhibited about 50% by glucose and by alanine, but not by glutamate. Since glucose also inhibited the rate of oxidation of acetate (Darling *et al.*, 1989a) and of octanoate and palmitate (Blum, 1990), it appears likely that the acetyl CoA derived from glycolysis or from alanine transamination and subsequent decarboxylation competes with the acetyl CoA derived from leucine or fatty acid oxidation. The lack of effect of added glutamate suggests that there was adequate OAA available from endogenous sources to condense with the acetyle CoA to be oxidized.

3.3. Arginine

Leishmania catabolizes arginine via the γ-guanidobutyramide pathway, which proceeds via several steps that include the formation of urea and, finally, succinate (Bera, 1987). The addition of glucose or glutamate increased the rate of urea production from arginine by promastigotes, while alanine and its nonmetabolizable analogue, α-aminoisobutyrate, inhibited urea formation (Blum, 1992). Leucine, proline, glycine, and lysine had no effect on the rate of urea formation. These findings have as yet no simple explanation, but serve to emphasize the complexity of interactions likely to determine the relative rates of consumption of individual amino acids and of glucose during growth. It should be noted that the rate of arginine catabolism, unlike that of leucine, alanine, or glutamate is only slightly inhibited by hyperosmolality, suggesting that the arginine transporter and the steps up to urea formation are insensitive to the signalling system (protein kinases? see Blum, 1993b) responsible for the inhibition of the catabolism of the above-mentioned amino acids.

Table II. Effect of Proline, Glutamate, Aspartate, and Alanine on $^{14}CO_2$ Formation from [6-^{14}C]Glucose

	Rate of [6-^{14}C]glucose oxidation (nmol h^{-1} (mg protein)$^{-1}$)		
Addition	305 mOsm kg^{-1}	610 mOsm kg^{-1}	% Inhibition
—	35.1 ± 1.8	$23.3 \pm 1.7^{*c}$	33
Alanine ($n = 4$)	21.7 ± 1.7^{b}	$16.5 \pm 1.0^{***b}$	24
—	32.6 ± 2.6	$22.7 \pm 2.0^{***}$	30
Glutamate ($n = 4$)	26.1 ± 3.8^{b}	$15.6 \pm 2.0^{**c}$	40
—	39.7 ± 5.9	$26.8 \pm 2.6^{*}$	32
Aspartate ($n = 3$)	27.3 ± 33.7^{a}	$19.5 \pm 1.9^{*b}$	29
—	41.7 ± 1.2	$25.8 \pm 1.6^{***}$	38
Proline ($n = 4$)	41.5 ± 2.4	$27.0 \pm 2.0^{**}$	35

*,**,*** $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, for hyperosmotic vs. iso-osmotic conditions.

[a,b,c] $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, for the presence of an amino acid vs. its absence at a given osmolality.

L. donovani promastigotes from late log phase cultures were collected, washed, and resuspended in HBSS$^-$ as described in Table I, and incubated with 3 mM [6-^{14}C]glucose in the presence or absence of 3 mM of the indicated unlabeled amino acids.

4. EFFECT OF SELECTED AMINO ACIDS ON GLUCOSE OXIDATION

In view of the enhancement of alanine oxidation by glutamate, proline, and aspartate, it was of interest to examine the effects of these three amino acids and of alanine on the rate of glucose oxidation. Table II shows that alanine, glutamate, and aspartate partially inhibited the rate of oxidation of [6-^{14}C]glucose by *L. donovani* promastigotes, but proline had no effect. Alanine also inhibits the rate of glucose oxidation by *L. braziliensis* promastigotes (Darling *et al.*, 1989a). The inhibitory effect of alanine might be explained merely as a consequence of the dilution of the labeled pyruvate formed by glycolysis with unlabeled pyruvate formed by alanine transamination. Glutamate and aspartate, however, enter the Krebs cycle as 2-KG and OAA, respectively, and could increase the rate of pyruvate utilization. If there was any effect of these two amino acids on the rate of glucose oxidation, one might therefore expect an increase rather than the observed inhibition. If the oxidation of glutamate or aspartate in the presence of glucose increased the ATP/ADP ratio, one might expect an inhibition of pyruvate kinase activity, since the activity of this enzyme isolated from *T. brucei* is inhibited by ADP (Barnard and Pedersen, 1988), and one or both of the pyruvate kinases (Ponte-Sucre *et al.*, 1993) of *L. donovani* are likely to behave in a similar manner. The failure of proline to inhibit glucose oxidation might then arise—at least in part—because [U-^{14}C]proline is oxidized to $^{14}CO_2$ at slightly less than half the rate at which [1-^{14}C]glutamate is oxidized (unpublished data). The tentativeness of this explanation emphasizes the need for further studies on the control of intermediary metabolism in these cells.

It should be noted (compare Table I with Table II) that hyperosmolality inhibits the oxidation of [6-^{14}C]glucose to only about half the extent to which it inhibits that of [U-^{14}C]alanine. This is consistent with the hypothesis (Blum, 1993b) that the signalling mechanism by which hyperosmotic stress affects metabolism includes an inhibition of the transport and/or transamination of alanine, as briefly discussed above.

5. MANNAN AND THE GLYOXYLATE CYCLE

Several lines of evidence indicated the presence of an energy reserve in at least some Trypanosomatidae: (1) whereas *T. brucei* bloodstream forms survive for only about 10–15 min in a buffer without glucose, the procyclic forms survive for much longer times (Opperdoes, personal communication); (2) whereas the rate of oxygen consumption (Q_{O_2}) of *L. donovani* promastigotes taken from late log phase cultures declines steadily from the time they are placed in a substrate-free buffer, and they remain fully motile for at most one hour, cells from a 3-day stationary phase culture maintain their Q_{O_2} and motility for many hours (Keegan and Blum, 1992); (3) *L. donovani* and, in some experiments, *L. major* promastigotes incubated for 1 h under an atmosphere of 95% N_2/5% CO_2 with glucose as sole exogenous carbon source release more carbons as acetate, pyruvate, succinate, D-lactate, and alanine than the number of carbons consumed (Darling *et al.*, 1989b; Walsh and Blum, 1991); (4) *L. mexicana* suspended in a non-nutrient buffer had almost the same Q_{O_2} as when suspended in growth medium (Coombs, 1981); (5) NMR studies indicate the presence of a carbohydrate in both the promastigote and amastigote forms of *L. m. pifanoi* (Rainey and MacKenzie, 1991).

In their studies on the glyoxylate cycle, Simon *et al.* (1978) found a substance that was precipitable by ethanol and which they assumed to be glycogen. Keegan and Blum (1992), however, could not demonstrate the presence of glycogen in *L. donovani*, but instead found that *L. donovani* promastigotes contained an ethanol-precipitable carbohydrate composed primarily, if not exclusively, of mannose residues. Although mannans had previously been reported to be present in *Herpetomonas samuelpessoai* and in several species of *Crithidia* (Previato *et al.*, 1982; Gorin *et al.*, 1979; Gottlieb, 1978; see also Marr, 1980), no experiments had been performed to ascertain whether they might act as an energy reserve. The mannan content of *C. fasciculata* declined with culture age (Gorin, 1979), but that of *L. donovani* increased with culture age (Keegan and Blum, 1992), consistent with the observation that promastigotes from 3-day stationary phase cultures maintained both motility and Q_{O_2} for much longer times than cells from late log phase cultures. Experiments were then performed in which 3-day stationary phase cells were incubated in buffer for 3 h in the presence and absence of glucose. In the absence of glucose, the mannan content dropped from about 19 to 8 μg/mg protein. This decrease did not occur in the presence of glucose and was partially prevented by the presence of alanine or glutamate (Keegan and Blum, 1992). These experiments support the view that in *L. donovani* mannan acts as a storage carbohydrate.

About 15 years ago, Mukkada and his coworkers (Simon *et al.*, 1978; Mukkada, 1977) presented evidence that isocitrate lyase and malate synthase, the two enzymes of the glyoxylate cycle, were present in several species of *Leishmania*. This cycle bypasses the oxidative decarboxylation steps at isocitrate dehydrogenase and 2-ketoglutarate dehydrogenase (see Fig. 1) and thus allows replenishment of Krebs cycle dicarboxylic acid intermediates from 2-carbon sources such as acetate and fatty acids. Simon *et al.* (1978) showed that label from ^{14}C-acetate was incorporated into an ethanol precipitable material (i.e., mannan) and thus demonstrated that the glyoxylate cycle could function in "gluconeogenesis." It has now been shown that label from [1-^{14}C]laurate is also incorporated into mannan (Keegan and Blum, 1993). Following the malate synthase-catalyzed reaction of acetyl CoA with the glyoxylate formed by the isocitrate lyase reaction, the malate so produced is shown in Fig. 1 as returning to the glycosome where it can be converted to OAA and then PEP via malate dehydrogenase and PEPCK. The subsequent path from PEP to the mannosyl residues of the mannan is at present unknown. Fructose-1,6-bisphosphatase(FBPase) has been reported to be present in several species of *Leishmania* (Martin *et al.*,

1976) and in *Trypanosome cruzi* (Adroher *et al.*, 1987). In *L. mexicana*, FBPase activity is present in both promastigotes and amastigotes, with much of the activity in the glycosome-enriched fraction (Coombs *et al.*, 1982). In view of the high concentration of phosphofructokinase (PFK) in glycosomes and its insensitivity to regulation by fructose-2,6-bisphosphate (Van Schaftingen *et al.*, 1985), it is difficult to envision how FBPase and PFK could both be located in the same compartment (as shown for convenience in Fig. 1) without excessive futile cycling. Since most of the glucose phosphate isomerase and appreciable triosephosphate isomerase and glyceraldehyde phosphate dehydrogenase activities occur in the cytoplasm of promastigotes (Hart and Opperdoes, 1984), it is possible that the "mannoneogenic" pathway is, indeed, cytosolic, but at present its localization is unknown. After the formation of F-6-P by the FBPase (whatever its localization), it must be converted to a mannosyl residue and incorporated into mannan. Although the enzymes involved in this pathway have not yet been characterized in *Leishmania*, it is known that the incorporation of [2-^{14}C]acetate into mannan is stimulated by glutamate and inhibited by hyperosmolality (Keegan and Blum, 1993). Whether this regulation occurs at the level of the glyoxylate cycle or the "mannoneogenic" pathway remains to be determined.

The utilization of the mannan presumably begins with hydrolysis by a β-mannanase, as in some bacteria and fungi (Arcand *et al.*, 1993), but studies on the biosynthetic or degradative pathways have yet to be performed.

6. EFFECTS OF CULTURE AGE ON INTERMEDIARY METABOLISM

During the growth of *Leishmania* in axenic culture, the composition of the culture medium changes (Hart and Coombs, 1982, and references therein). With increasing time in the stationary phase there are also marked increases in infectivity (Sacks and Perkins, 1985, and references therein) and changes in the levels of many enzymes, including those of intermediary metabolism (Mallinson and Coombs, 1986, 1989). These changes continue during transformation to the amastigote form. We shall here make no attempt to review the large amount of data already published on the various changes that occur, but will focus on a few observations relevant to the bioenergetics of the cells.

The rates of respiration of *L. mexicana* amastigotes, whether measured in fresh growth medium or in buffer, were about the same on a per mg protein basis as those of promastigotes (Hart *et al.*, 1981). For *L. major*, however, the metacyclic forms (i.e., cells taken from late stationary phase cultures) had a 50% lower Q_{O_2} when resuspended in fresh medium (Mallinson and Coombs, 1989). *L. donovani* taken from 3-day stationary phase cultures consumed O_2 at the same rate as promastigotes taken from log phase cultures when incubated in buffer alone, and a 30% lower rate in buffer containing glucose (Keegan and Blum, 1992). These results suggest that depending on the species, growth conditions, and time in stationary phase, a decrease in overall energy production rate may occur, but if so, only in late stationary phase cells. Consistent with this is the decreased level of succinic dehydrogenase in *L. mexicana* amastigotes as compared to promastigotes (Coombs *et al.*, 1982).

The ability of washed promastigotes to oxidize fatty acids increases with culture age (Blum 1990), and fatty acids are more important substrates for amastigotes than for promastigotes (Hart and Coombs, 1982). In accord with these observations, higher activities of several enzymes of the β-oxidation pathway (Coombs *et al.*, 1992) and much higher levels of free carnitine and carnitine esters (Cooper and Coombs, 1985) are found in amastigotes than in promastigotes. *L. major* metacyclic forms contain many more lipid globules than do promastigotes from log phase cultures (Mallinson and Coombs, 1989). Similarly, *L. braziliensis* promastigotes that are transferred from 26°C to 34°C, which initiates a partial transformation toward an amastigote-like form, also show an increase in lipid droplet content (Stinson *et al.*, 1989). Simultaneous measurements of the rates of utilization of lipids and of mannan by washed cells as a function of culture age could provide valuable information concerning the relative contributions of these two classes of energy reserves.

Decreases in the rates of oxidation of ^{14}C-labeled glucose, alanine, and acetate with increasing culture age have been reported for *L. major* (Keegan and Blum, 1990), and of ^{14}C-alanine for *L. braziliensis (Keegan et al.*, 1987). The rates of oxidation by *L. donovani* of ^{14}C-labeled leucine, proline, and aspartate (Blum, 1991, and unpublished data) and of arginine catabolism to urea (Blum, 1992) also decrease with increasing age of the culture from which the cells are collected. Figure 2 shows the decline in rate of oxidation of [1-^{14}C]glutamate and

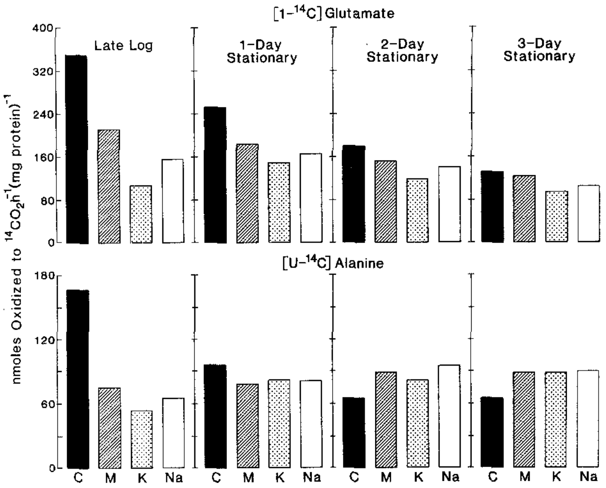

Fig. 2. Four flasks of *L. donovani* were grown to late log, 1-, 2-, and 3-day stationary phase. On each day the contents of one flask were centrifuged and the cells washed and resuspended in HBSS$^-$. Aliquots of the washed cells were then incubated in triplicate for 1 h in the presence of 4 mM [U-^{14}C]alanine or 2 mM [1-^{14}C]glutamate in HBSS$^-$ (iso-osmotic, 305 mOsm kg^{-1}; solid bar, C) or in HBSS$^-$ containing 305 mM mannitol (bars labeled M), 152.5 mM KCl (bars labeled K), or 152.5 mM NaCl (bars labeled Na), and $^{14}CO_2$ production was then measured. The means of each set of triplicate measurements, which generally differed by less than 10%, are shown for each condition.

of [U-^{14}C]alanine with decreasing culture age, and also shows the effects of hyperosmolality at each culture age. For glutamate, the inhibitory effect of hyperosmolality—whether caused by addition of NaCl, KCl, or mannitol to the buffer—decreases with culture age, becoming very small in cells taken from 3-day stationary phase cultures. The effect of hyperosmolality on alanine oxidation is, however, different than for glutamate. After one day in stationary phase, hyperosmolality caused only a small inhibition of the rate of alanine oxidation, while in cells from 2-day and 3-day stationary phase cultures the rate was increased. Thus, in addition to the decreases in rate of oxidation of most substrates tested (except fatty acids, as discussed above), increasing culture age also changes the response to hyperosmolality, especially for alanine. Studies using protein kinase inhibitors suggest that protein kinases play an important role in regulating at least some of the change in metabolism and response to hyperosmolality that occur with increasing culture age (Blum, 1993b). With the advent of methods for the transformation of axenic promastigotes of several species of *Leishmania* into metacyclic forms (Bates and Tetley, 1993) and into amastigote-like forms (see e.g. Joshi *et al.*, 1993), it may be expected that a deeper understanding of the changes in metabolism and bioenergetics that occur as promastigotes age and develop into metacyclic forms and then amastigotes will emerge.

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