Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 7129–7133, October 1987
Cell Biology

Products of *Leishmania braziliensis* glucose catabolism: Release of D-lactate and, under anaerobic conditions, glycerol*

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Communicated by Robert L. Hill, June 25, 1987

ABSTRACT ***Leishmania braziliensis panamensis*** **promastigotes were incubated with glucose as the sole carbon source. About one-fifth of the glucose consumed under aerobic conditions was oxidized to CO_2. Nuclear magnetic resonance studies with [1-^{13}C]glucose showed that the other products released were succinate, acetate, alanine, pyruvate, and lactate. Under anaerobic conditions, lactate output increased, glycerol became a major product, and, surprisingly, glucose consumption decreased. Enzymatic assays showed that the lactate formed was D(−)-lactate. The release of alanine during incubation with glucose as the sole carbon source suggested that appreciable proteolysis occurred, consistent with our observation that a large amount of ammonia was released under these conditions. The discoveries that D-lactate is a product of *L. braziliensis* glucose catabolism, that glycerol is produced under anaerobic conditions, and that the cells exhibit a "reverse" Pasteur effect open the way for detailed studies of the pathways of glucose metabolism and their regulation in this organism.**

Trypanosomatids are a metabolically diverse group of protozoans that form, in addition to CO_2, several incompletely oxidized products under aerobic conditions. These products, some of which are specific to certain species, include ethanol, succinate, acetate, pyruvate, and lactate. The relative amounts of these products change markedly under anaerobic conditions. *Trypanosoma brucei*, for example, catabolizes glucose almost exclusively to pyruvate aerobically but forms equimolar pyruvate and glycerol anaerobically (1).

Little is known concerning the products of glucose catabolism by *Leishmania* spp. under either aerobic or anaerobic conditions. Studies on cells growing in complex medium indicate that CO_2, succinate, acetate, pyruvate, lactate, and alanine are produced, as well as several other amino acids (2–7). In studies using enzymatic assays, no accumulation of lactate during growth has been noted (2, 6), but some studies using chemical assay methods have reported the production of lactate, aerobically (ref. 3; see, however, ref. 4) and anaerobically (8).

Studies using enzymatic or chemical assay methods are limited to detecting those compounds presumed to be present. NMR spectroscopy of the products formed by cells incubated in the presence of a single ^{13}C-labeled substrate, however, will detect all major water-soluble products simultaneously. This technique has been used to study the anaerobic glucose metabolism of two trypanosomatids, revealing the unexpected formation of alanine along with pyruvate and glycerol by *T. brucei* (9, 10) and of glycerol as well as ethanol, succinate, and an unidentified compound by *Crithidia fasciculata* (11). Here we report that *Leishmania braziliensis* promastigotes incubated with [1-^{13}C]glucose as sole carbon source form succinate, acetate, pyruvate, alanine, and as demonstrated enzymatically, D-lactate. To our knowledge, this is the first report of the production of D-lactate by any parasitic protozoan. Under anaerobic conditions, glycerol becomes a major product, and D-lactate formation increases markedly while glucose consumption decreases.

MATERIALS AND METHODS

Materials. D-[1-^{13}C]Glucose (99% atom enriched) was from Omicron Biochemicals (Ithaca, NY). Glucose reagent, enzymes, D(−)-lactate, L(+)-lactate, hydrazine hydrate, growth medium components, and fetal bovine serum were from Sigma. All other chemicals were of reagent grade.

Growth of Organisms. *Leishmania braziliensis panamensis* (MHOM/PA/82/WR470) was grown at 26 ± 0.5°C in 500-ml Erlenmeyer flasks as described (12). Fifty milliliters of medium was inoculated with 3 ml of stock culture. Two days later, 50 ml of fresh medium was added, and the flasks were put in a 26°C rotary shaker bath. Cells in early stationary phase were harvested 16–24 hr after adding the fresh medium.

Incubations and Assays. Cells were collected by centrifugation at 1400 × *g* for 4 min in a Sorvall RC-5 centrifuge. The medium was decanted, and the pellet was resuspended in buffer A (10 mM Hepes plus Hanks' balanced salt solution without glucose or phenol red, pH 7.1) or buffer B (identical to buffer A except that 10 mM Na_2HPO_4/10 mM KH_2PO_4 was substituted for Hepes). The cells were centrifuged again, and the buffer was decanted. The resulting pellet (21–34 mg of protein) was resuspended in 2.6–3.8 ml of buffer A or buffer B containing 5.55 mM [1-^{13}C]glucose. One drop was examined under phase-contrast microscopy, samples were taken for protein measurement, and the remainder was pipetted into a 50-ml Erlenmeyer flask and incubated for either 60 or 90 min at 26°C in a shaker bath at 70 oscillations per min. Halfway through the incubation, enough 75 mM [1-^{13}C]glucose in buffer B (pH 9.0) was added to increase the glucose concentration by 2.78 mM and to minimize decreases in pH due to the release of organic acids. For anaerobic experiments, the cells were resuspended in buffer that had been bubbled with N_2 for 10 min, and the incubation flask was flushed with N_2 and capped. Triplicate 0.1-ml samples were taken at the end of the incubation periods, and glucose and lactate were measured. The remainder was centrifuged at 2300 × *g* for 2.5 min at 5°C. The supernatant (extracellular) fraction was collected and deproteinized with 160 μl of 13% (vol/vol) perchloric acid. The pellet was washed once in ice-cold buffer and centrifuged as above, the supernatant was decanted, and then 1.0 ml of 13% (vol/vol) perchloric acid was added to release the intracellular contents. Both the extracellular and intracellular fractions were then centrifuged at 2300 × *g* for 10 min, and the supernatants were

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*A preliminary report of these results has been presented at a meeting of the Society of Protozoologists, July 7–11, 1987, Urbana–Champaign, IL.

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neutralized with KOH and centrifuged again. The final supernatants were filtered through a Swinnex-GS 0.22-μm Millipore filter and frozen until NMR measurement. Concomitant with some of the incubations for the NMR experiments, the rate of $^{14}CO_2$ production from [1-^{14}C]glucose was measured as described (13).

In experiments where glucose consumption and lactate or glycerol production were measured, the cells were washed as before and resuspended in buffer A, samples were taken for protein measurement, and the remaining cells were divided between a 50-ml Erlenmeyer flask and either a 125-ml or a 250-ml Erlenmeyer flask. An equal volume of buffer A containing 11.1 mM glucose (bubbled with N_2 for 10 min for anaerobic conditions) was added so that the initial incubation volume was 4.6–6.4 ml. The 50-ml flask was flushed with N_2 and capped, whereas the 125- or 250-ml flasks were left open. The cells were incubated at 26°C in a shaker bath. At 0, 30, 60, and 90 min, triplicate 0.45-ml samples were pipetted into 12-ml centrifuge tubes; each tube was covered with a marble and put in a boiling water bath for 60 sec and then placed in ice. The 50-ml flask was flushed with N_2 and recapped after taking samples. The chilled samples were centrifuged at 4°C for 10 min at 500 × *g*, and glucose and lactate or glycerol were measured in the supernatants. In separate aerobic experiments, ammonia and urea production were measured for cells in buffer A with and without 8.0 mM glucose. Alanine content in freshly resuspended cells was also measured.

Glucose was measured spectrophotometrically at 505 nm using a commercially available glucose reagent with oxidation of 4-aminoantipyrine to form a quinoneimine dye through coupled reactions catalyzed by glucose oxidase and peroxidase (Sigma procedure 315). L-Lactate was measured fluorimetrically (14). Samples were incubated with rabbit muscle L-lactate dehydrogenase and NAD at pH 9.0 for 2 hr at 34°C. Fluorescence was measured using a Farrand Optical (Valhalla, NY) A4 system filter fluorometer with a Corning 7-51 primary filter and a 3-73 secondary filter. D-Lactate was measured spectrophotometrically using D-lactate dehydrogenase from *Leuconostoc mesenteroides* and NAD (15). Alanine was measured using L-alanine dehydrogenase as described (16). Glycerol was measured spectrophotometrically by incubating samples for 2 hr at 25°C in an assay mixture containing glycerokinase, glycerol-3-phosphate dehydrogenase, NAD, and ATP in glycine/hydrazine buffer, pH 9.9 (17). Ammonia content was measured spectrophotometrically at 340 nm by the L-glutamate dehydrogenase-catalyzed reaction of ammonia with 2-oxoglutarate and NADH (Sigma procedure 170-UV). Urea was measured by incubating samples with diacetyl monoxime and measuring the absorbance at 520 nm (Sigma procedure 535). Protein was measured by the method of Lowry *et al.* (18) using bovine serum albumin as standard.

NMR Measurements. Samples for ^{13}C NMR were adjusted to ≈2.2 ml by the addition of ^2H_2O for field/frequency stabilization and placed in 10-mm (o.d.) sample tubes (Wilmad Glass, Buena, NJ). Spectra were taken at 22°C in the Fourier-transform mode at 125 MHz on a General Electric GN-500 spectrometer system, using a 45° flip-angle (8 μsec) and a 2.5-sec delay between acquisitions. Two-level WALTZ (19) ^{2}H decoupling was applied during the preacquisition and acquisition periods. The free induction decays were collected using quadrature phase detection in four-to-five blocks of 16,000 data points; each block was composed of 4000 acquisitions. The data sets were scaled, added, and apodized with a 4-Hz Gaussian filter function before Fourier transformation to give frequency domain spectra of 26,000-Hz width. A small amount of unlabeled dioxane, added to one of the samples, was used to calibrate the chemical shifts. Assignment of the peaks was based on standard tables (20) and by identification of the chemical shifts of the protons bonded to the ^{13}C-labeled sites using two-dimensional absorption-mode heteronuclear chemical shift correlation spectroscopy (21).

RESULTS

The ^{13}C NMR spectra of cells incubated in buffer A contained several signals due to the natural ^{13}C content of Hepes. Since these signals were in the range of some of the ^{13}C-labeled metabolites, buffer B, containing phosphate instead of Hepes, was used after it was established that the spectra obtained from cells in the two buffers were qualitatively identical (except for the signals due to Hepes) and that the rates of $^{14}CO_2$ production from [1-^{14}C]glucose, [1,3-^{14}C]glycerol, or [*U*-^{14}C]alanine were within 15% of the values obtained for the same cells incubated in buffer A (data not shown).

Fig. 1*A* shows a typical NMR spectrum obtained from the extracellular fraction of promastigotes incubated with [1-^{13}C]glucose as sole exogenous carbon source. In addition to the α and β anomers of glucose (data not shown), signals arising from C-3-labeled alanine, C-3-labeled pyruvate, C-3-labeled lactate, C-2-labeled acetate, and succinate labeled at C-2 and/or C-3 were identified in the upfield portion of the spectrum. In some of the spectra, small satellites due to ^{13}C–^{13}C scalar coupling were observed at the base of the succinate resonance. The coupling constant of 50.4 Hz is consistent with a coupling interaction involving a methylene-carboxyl group (22), and hence arises from a small amount of [1,2-$^{13}C_2$]succinate (or equivalently [3,4-$^{13}C_2$]succinate). Similar metabolite profiles were observed in each of four such experiments. In cells incubated with unlabeled glucose, these resonances were not observed. The carbon positions that are labeled are consistent with the known biochemistry of the organisms and with glucose metabolism through the Embden–Meyerhof pathway.

The production of labeled alanine from labeled glucose suggests proteolysis as a source of amino groups. Since *Leishmania* promastigotes excrete urea and ammonia during growth in complex medium (23, 24) and since glucose decreases endogenous protein degradation in *Leishmania* (25), we examined ammonia and urea production by *L. braziliensis* incubated aerobically in buffer A with and without 8.0 mM glucose. Ammonia production was 204 ± 60 nmol per mg of protein per hr (n = 4) in the absence of glucose and was decreased in the presence of glucose [ratio of rates of ammonia production ± glucose, 0.47 ± 0.21 (n = 4)]. The rate of urea production was too small to measure.

The ^{13}C NMR spectrum of the intracellular fraction (Fig. 1*B*) indicates the presence of most of the same labeled metabolites as are observed in the extracellular fraction, with several differences: (*i*) there is no significant pyruvate C-3 resonance; (*ii*) the ratio of labeled alanine to other metabolites is considerably higher for the intracellular pool; and (*iii*) there is a significant increase in the [2-^{13}C]alanine resonance, which is surprising since the labeling of the intra- and extracellular pools would in general be expected to be similar. This difference probably reflects a time-dependent increase in the production of the [2-^{13}C]alanine. Thus, the intracellular fraction largely represents the labeling of this pool at the end of the experiment, whereas the extracellular fraction represents the average of the excreted metabolites over the course of the incubation.

The intense alanine C-3 resonance observed suggests a large intracellular pool of this amino acid, as was also found for *Leishmania tropica* (26). This conclusion is supported by the observation that small but significant C-2 and C-3 alanine resonances are observed in the intracellular fraction of cells incubated with unlabeled glucose. The presence of a large intracellular pool of alanine was confirmed by measuring the

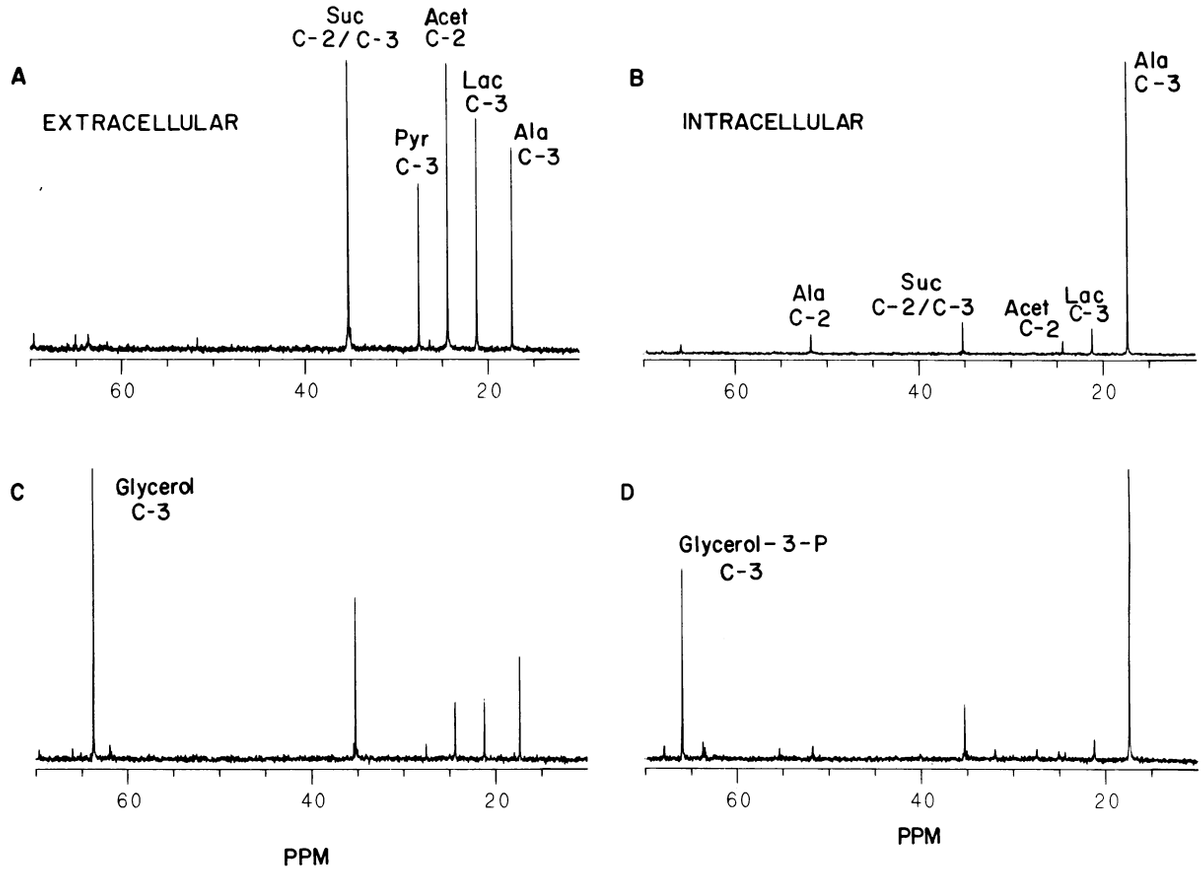

FIG. 1. Proton-decoupled ^{13}C NMR spectra of extra- and intracellular fractions of *L. braziliensis* incubated with [1-^{13}C]glucose at 26°C. Spectra for promastigotes incubated for 1 hr under aerobic conditions (*A* and *B*) and for 1.5 hr under anaerobic conditions (*C* and *D*). Spectra of extracellular fractions (*A* and *C*); spectra of intracellular fractions (*B* and *D*). Final glucose concentrations, determined enzymatically, were 0.88 mM and 2.76 mM for the aerobic and anaerobic experiments, respectively. The metabolites identified and their chemical shifts, in ppm, relative to dioxane (δ = 67.4 ppm) are as follows: 17.30 ppm, alanine C-3; 51.70 ppm, alanine C-2; 21.16 ppm, lactate C-3; 24.32 ppm, acetate C-2; 27.50 ppm, pyruvate C-3; 35.19 ppm, succinate C-2 and/or C-3; 63.64 ppm, glycerol C-1,(3); and 65.88 ppm, glycerol-3-phosphate C-3. Suc, succinate; Acet, acetate; Pyr, pyruvate; Lac, lactate; Glycerol-3-P, glycerol-3-phosphate.

alanine content [0.234 ± 0.005 μmol/mg of protein (n = 2)] of freshly washed promastigotes.

Ellipsoidal forms, derived from promastigotes preincubated in growth medium for 6 hr at 34°C (12), had spectra qualitatively identical to those from promastigotes for both the extracellular and intracellular samples (data not shown).

As a consequence of the conflicting reports of lactate formation by *Leishmania* (see above), confirmation of the NMR identification by an enzymatic method seemed to be indicated. A fluorimetric assay sensitive to 5 μM L-lactate was negative, in apparent conflict with the ^{13}C NMR data. This discrepancy was resolved by use of an assay for D-lactate, which indicated the presence of ≈1 mM D-lactate in the extracellular fractions analyzed by NMR. D-Lactate (≈60 μM) was also present in medium obtained from early stationary and stationary phase cultures. The assay used is highly specific for D-lactate; no increase in A_{340} was seen with up to 5 mM L-lactate.

^{13}C NMR spectra of the extracellular fraction from promastigotes incubated anaerobically with [1-^{13}C]glucose (Fig. 1*C*) showed most of the same labeled metabolites as cells incubated aerobically. The primary difference was the large ^{13}C resonance observed at 63.6 ppm, which was assigned to [1,3-^{13}C]glycerol (20). This was confirmed by an enzymatic assay, which indicated ≈0.7 mM glycerol. To our knowledge, this is the first time that glycerol has been reported as a product of *Leishmania* metabolism, although glycerol production has been observed under anaerobic conditions in *C. fasciculata* (11) and in several species of trypanosomes (27, 28).

In the intracellular fraction of anaerobically incubated cells, a resonance at 65.9 ppm, which is split by 4.7 Hz into a doublet, is observed, and this is assigned to [3-^{13}C]glycerol-3-phosphate. The only glycolytic intermediate observed in *T. brucei* under anaerobic conditions is also glycerol-3-phosphate (9), and a large increase in the concentration of this metabolite is observed in anaerobic or salicylhydroxamic acid-treated *T. brucei* (29, 30).

Relative signal intensities cannot always be used directly for quantification of metabolite concentrations (9), so enzymatic assays were used to quantitate the rates of glucose consumption and D-lactate production, and, in separate experiments, the rates of glucose consumption and glycerol production. No attempt was made to establish a complete carbon or redox balance. Although the NMR signal from glycerol was larger than that from D-lactate (Fig. 1*C*), enzymatic assays showed comparable rates of production of glycerol and D-lactate under anaerobic conditions (Table 1). This suggests preferential conversion of carbons 1–3 of glucose into glycerol, as observed in *T. brucei* (9). Glucose consumption and D-lactate production were linear with time for up to 90 min in cells incubated aerobically or anaerobically (data not shown). Glycerol production was linear for 60 min in cells incubated anaerobically. The rate of D-lactate production was >5-fold higher in cells incubated anaerobically than in controls incubated aerobically, but the rate of glucose consumption decreased by ≈50% (Table 1). Thus the

Table 1. Consumption of glucose and production of D-lactate, glycerol, and CO_2 by *L. braziliensis* promastigotes

	Compound, nmol per mg of protein per hr			
	Glucose	D-Lactate	Glycerol	CO_2
Experiment 1				
Aerobic	477 ± 43 (4)	11.3 ± 0.3 (3)		
Anaerobic	208 ± 6 (3)	66.9 ± 8.6 (3)		
Experiment 2				
Aerobic	620 ± 69 (3)		2.1 ± 2.5 (3)	
Anaerobic	312 ± 11 (3)		50 ± 20 (3)	
Experiment 3				
Aerobic				103.7 ± 8.4 (6)
Anaerobic				NM

Cells were incubated under aerobic and anaerobic conditions in buffer A containing 5.55 mM glucose. The rates of glucose consumption and D-lactate and glycerol production were calculated from the linear slopes. The rate of $^{14}CO_2$ production was measured from [1-^{14}C]glucose (30 dpm/nmol). Results are the mean ± SD of the number of experiments shown in parentheses. NM, not measured.

percent of glucose going into lactate, assuming two lactate molecules per glucose molecule and neglecting any production of lactate from endogenous sources, increases from 1.2% aerobically to 16.1% anaerobically. Almost no glycerol was formed aerobically, but ≈8% of the glucose utilized anaerobically appeared as glycerol (assuming two glycerol molecules per glucose molecule consumed). CO_2 accounts for about one-fifth of the C-1 of glucose that is utilized aerobically (Table 1).

DISCUSSION

To our knowledge, *L. braziliensis* is the only protozoan parasite known so far to produce D-lactate. D-Lactate is produced anaerobically by a number of species including certain bacteria (31), unicellular algae (32), spiders (33), and sperm of the giant octopus (34). D-Lactate can be formed by D-lactate dehydrogenase, which is found in a large variety of prokaryotes and eukaryotes, including the flagellate *Euglena* (35), or by pyruvate reductase, as described in *Chlamydomonas reinhardtii* (32). It can also be formed by the action of a dual-enzyme system, glyoxalase I and glyoxalase II, using methylglyoxal as substrate and glutathione as coenzyme (15). Methylglyoxal can be formed from dihydroxyacetone phosphate by methylglyoxal synthase, present in certain bacteria such as *Escherichia coli* (36). It is presently unknown which of these pathways function in *L. braziliensis*. Martin *et al.* (37) were unable to detect any lactate dehydrogenase activity (using D,L-lactate as substrate) in cell-free extracts of four species of *Leishmania*, but Kreutzer *et al.* (ref. 38 and references cited therein) reported this activity (also using D,L-lactate) in many species of *Leishmania*.

Leishmania are able to survive (but not grow) for several days under anaerobic conditions in complex medium (39, 40). However, little is known about their metabolism under such conditions. The present results show that survival may be mediated at least in part by increased production of D-lactate and glycerol, both of which regenerate NAD from the NADH produced by glycolysis. The large increase in intracellular concentration of glycerol-3-phosphate suggests that the pathway for glycerol formation involves L-α-glycerophosphate dehydrogenase and glycerophosphate phosphatase, enzymes known to be present in four species of *Leishmania* (37), though a reversal of the glycerol kinase reaction as in *T. brucei* (ref. 1 and references cited therein) is also possible.

L. braziliensis promastigotes reduce glucose consumption when placed under anaerobic conditions. This "reverse" Pasteur effect was not due to parasite death, since cells examined (aerobically) after 90 min of anaerobiosis displayed vigorous motility and had normal shape. A lack of a Pasteur effect has been noted in *Leishmania donovani* (8) and several *Trypanosoma* spp. (27, 28, 41). The only other organisms known to us that exhibit a reverse Pasteur effect are *Chlorella* (42), *Trichomonas vaginalis* (43), and *Brugia pahangi* microfilariae (44). The microfilariae were less motile under anaerobic conditions, and Rew and Saz (44) suggested that the lower energy expenditure might account for the reduced glucose consumption. *Leishmania mexicana* promastigotes also show decreased motility anaerobically (40), but it is not likely that flagellar motility accounts for an appreciable fraction of the energy expenditure of flagellates. Since proline transport is abolished by anoxia and since uptake of 2-deoxyglucose is inhibited by rotenone or antimycin A (45), it is possible that the reverse Pasteur effect results from inhibition of glucose transport.

Succinate is a major product of *L. braziliensis* glucose metabolism under aerobic conditions. It is also the major product of *Trypanosoma cruzi*, *C. fasciculata*, and *L. mexicana* when grown aerobically in complex media (2). The ^{13}C NMR labeling pattern of succinate obtained in the present study can be interpreted as resulting from condensation of [2-^{13}C]acetyl CoA with oxaloacetate followed by forward movement through the Krebs cycle, yielding succinate labeled at C-2 or C-3. Condensation of the [2-^{13}C]acetyl CoA with [3-^{13}C]oxaloacetate would be expected to yield [1,3-$^{13}C_2$]succinate (or equivalently [2,4-$^{13}C_2$]succinate). The absence of this signal may be explained by the difficulty in observing the very small two-bond ^{13}C coupling, and the low probability of obtaining doubly labeled molecules as a consequence of the label dilution that results from the formation of two triose molecules from the singly labeled glucose.

Succinate is also a major product of anaerobic glucose metabolism by *L. braziliensis*, as it is for *C. fasciculata* (11) and *L. donovani* (8). In *C. fasciculata*, formation of succinate anaerobically appears to be through carboxylation of phospho*enol*pyruvate and conversion of the oxaloacetate so formed through malate and fumarate to succinate (11). The observation of a small amount of [1,2-$^{13}C_2$]succinate both aerobically and anaerobically can also be explained by this pathway. Reincorporation (by phospho*enol*pyruvate carboxykinase) of $^{13}CO_2$ released during the incubation would yield [3,4-$^{13}C_2$]oxaloacetate that could be converted to [1,2-$^{13}C_2$]succinate through malate and fumarate. Alternatively, flux through multiple turns of the Krebs cycle in the forward direction with the introduction of label through acetyl CoA at each turn could explain this labeling pattern. Further work will be required to distinguish between these possibilities.

The major 3-carbon compounds produced aerobically from [1-^{13}C]glucose are pyruvate, D-lactate, and alanine, with alanine usually predominating. *L. donovani* promastigotes

also incorporate more label from glucose into alanine than into all the other amino acids combined (46), and formation of alanine from glucose has also been shown in *T. brucei* (9). The necessary transaminases are present in *Leishmania* (26, 47–49). The amino groups appear to be derived from amino acids obtained by intracellular protein degradation, perhaps of a particular group of 30- to 60-kDa proteins (25). The rate of proteolysis increases markedly when cells are incubated without nutrients and is inhibited by 20 mM glucose (25). The present results confirm the observation that glucose reduces the rate of proteolysis, as judged by ammonia release rather than by degradation of L-[^{3}H]leucine-labeled proteins.

The release of acetate as a product of intermediary metabolism is a common but poorly understood phenomenon in parasites. Several possible pathways for the formation of acetate have been studied in *Entamoeba histolytica* (50), *Trichomonas foetus*, and *Trichomonas vaginalis* (51), and a nonspecific acetyl esterase has been reported in many protists (52). At present, however, nothing is known about the pathway(s) of acetate formation in *Leishmania*.

The use of NMR to study products of *L. braziliensis* glucose catabolism has led to the identification of D-lactate and the demonstration that glycerol is formed under anaerobic conditions. Detection of D-lactate might be useful in the diagnosis of leishmaniasis, especially in visceral leishmaniasis where large parasite burdens and anaerobic conditions may result in significant production of D-lactate. Much remains to be learned about the pathways involved in the formation of these and other products.

Note Added in Proof. We have found that ultrasonically disrupted promastigotes catalyze the conversion of dihydroxyacetone phosphate to D-lactate and that intact cells convert methylglyoxal to D-lactate at a high rate.

T.N.D. was supported by the Medical Scientist Training Program from the National Institutes of Health (Grant 5T32-GM07171).

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