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SODIUM STIBOGLUCONATE (PENTOSTAM) INHIBITION OF GLUCOSE CATABOLISM VIA THE GLYCOLYTIC PATHWAY, AND FATTY ACID β-OXIDATION IN *LEISHMANIA MEXICANA* AMASTIGOTES

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Abstract—The biochemical mechanism of action of antimony (Sb) in pentavalent form complexed to gluconic acid (sodium stibogluconate)—the drug of choice for the leishmaniases—has been only slightly investigated. We recently reported that, in stibogluconate-exposed *Leishmania mexicana* amastigotes, there is a dose-dependent decrease in the ATP/ADP ratio [Berman *et al.*, *Antimicrob. Agents Chemother.* **27**, 916 (1985)]. To investigate mechanisms by which ADP phosphorylation to ATP might be inhibited, stibogluconate-exposed amastigotes were incubated with [^{14}C]glucose, fatty acid, or acetate, and $^{14}CO_2$ production was determined. In organisms exposed to 500 μg Sb/ml, formation of $^{14}CO_2$ from [6-^{14}C]glucose and [1-^{14}C]palmitate was inhibited 69 and 67% respectively. In comparison, formation of $^{14}CO_2$ from [1-^{14}C]glucose and [2-^{14}C]acetate was inhibited <15%. These results suggest that glucose catabolism via glycolytic enzymes and fatty acid β-oxidation, but not glucose metabolism via the hexosemonophosphate shunt or the citric acid cycle, is specifically inhibited in stibogluconate-exposed *Leishmania mexicana* amastigotes. Inhibition of these pathways suggests a mechanism for the inhibition of ADP phosphorylation previously reported.

The leishmaniases are visceral and cutaneous diseases of widespread tropical and semi-tropical distribution. The disease results from multiplication of the amastigote form of the parasite within the macrophages of the viscera or the skin. Although treatment with antimonials may have been used by the ancient Greeks [1], and pentavalent antimonials such as sodium stibogluconate (Pentostam) have been the drugs of choice since the 1940s, in early 1985 Mottram and Coombs still could write that "there are no reports on the mechanism of antileishmanial antimonials" [2]. Other clinical antileishmanial agents are pentamidine and amphotericin B; the major experimental agents are allopurinol ribonucleoside, ketoconazole, and the 8-aminoquinoline WR 6026. Except for amphotericin B and ketoconazole, the mechanisms of which are thought to be comparable to those against fungi [3], biochemical pathways in *Leishmania* inhibited by these agents have not been identified.

We recently reported that, when amastigotes of *Leishmania mexicana* are exposed to 150–500 μg Sb in the form of stibogluconate for 4 hr *in vitro*, there is a decrease in viability and in synthesis of RNA and DNA. In addition, there is a decrease in net ATP formation but an increase in net AMP and ADP formation. Organisms exposed to 150 μg Sb/ml exhibit a slight change in these variables, whereas organisms exposed to 500 μg Sb/ml demonstrate an approximately 40–60% change [4]. We therefore postulated that the antileishmanial mechanism of stibogluconate may be associated with the inability of the amastigotes to phosphorylate ADP, a major end result in bioenergetics. Here we present evidence that glucose catabolism and β-oxidation are specifically inhibited in stibogluconate-treated *L. mexicana* amastigotes.

MATERIALS AND METHODS

Exposure of L. mexicana *amastigotes to stibogluconate and to radiolabels.* Amastigotes of *L. mexicana* WR 227 were purified from infected J774 tumor macrophages (American Type Culture Collection, Rockville, MD) by the method of Chang [5] with slight modifications, precisely as previously reported [4]. The washed, pure amastigotes were suspended (25–30 × 10^6 organisms/ml) in medium [RPMI-1640 (GIBCO, Grand Island, NY) with 10% heat-inactivated fetal calf serum] for 4 hr at 35° and pH 7.4 (or pH 4.5 where noted) with 0, 150, 300, or 500 μg antimony (Sb) in the form of Pentostam (Burroughs Wellcome, Inc., England) per ml. Aliquots of the organisms were exposed to 0.4 μCi [^{14}C]leucine (330 mCi/mmole, New England Nuclear, Boston, MA) per ml as well as to stibogluconate to ascertain inhibition of protein synthesis due to Sb. After being washed with 10 vol. of phosphate-buffered saline (PBS; GIBCO) by centrifugation (1000 *g* × 10 min), the stibogluconate [^{14}C]leucine-treated amastigotes were allowed to settle into glass filter paper, washed with water and methanol under suction, dried, and counted in a

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scintillation counter. Protein synthesis was used as an internal control to verify that the drug was active in each experiment and to permit comparison with our previous report. In the present work, organisms exposed to 150, 300 and 500 μg Sb/ml had means of 84, 62 and 44%, respectively, of control incorporation of leucine into protein (4024 cpm/10^8 amastigotes). The rest of the amastigotes exposed to 0–500 μg Sb/ml were utilized to determine generation of CO_2 from radiolabeled precursors (glucose, acetate, or fatty acid). After stibogluconate exposure, the organisms were resuspended in PBS, and 5–10 × 10^6 organisms in 75 μl PBS were added to wells in which 75 μl of radiolabeled precursor had already been added. The wells were covered with filter paper coated with $Ba(OH)_2$ for 30 min, treated with 0.5 vol. of 15% trichloroacetic acid, and covered again with the filter paper for 10 min. The filter paper containing $BaCO_3$ that resulted from the reaction of the barium with liberated CO_2 was dried and counted in a gas-flow counter [6]. In experiments in which incorporation of radiolabeled energy precursors into the organisms was determined, the organisms were exposed to precursor for 30 min, then washed × 4 by centrifugation (9000 *g* × 2 min) and scintillation counted.

Chromatography. Net formation of organic acids was investigated in organisms exposed to radiolabeled precursors. The trichloroacetic acid treated suspension of organisms was centrifuged (9000 *g* × 2 min), and the TCA-supernatant fraction was chromatographed on PEI cellulose plates (Beckman, Nutley, NY) with butanol–H_2O–formic acid (40:50:10) as mobile phase for 6 hr, or on silica IB2F plates (Baker, Phillipsburg, NJ) with petroleum ether–diethyl ether–formic acid (28:12:1) as mobile phase for 2 hr. The sections of the chromatogram corresponding to simultaneously chromatographed external standards were cut out and counted in a scintillation counter.

Materials. [1-^{14}C]Glucose (57 mCi/mmole), [6-^{14}C]glucose (9.5 mCi/mmole), [U-^{14}C]glucose (258 mCi/mmole), [1-^{14}C]palmitic acid (58 mCi/mmole), [U-^{14}C]palmitic acid (800 mCi/mmole), [2-^{14}C]acetate (1.8 mCi/mmole), [U-^{14}C]acetate (54.7 mCi/mmole), and [1,4-^{14}C]succinic acid (53.5 mCi/mmole) were purchased from New England Nuclear. Cold chromatography standards were purchased from the Sigma Chemical Co., St. Louis, MO.

RESULTS

The formation of CO_2 from precursors in control *L. mexicana* amastigotes (organisms that had not been pretreated with stibogluconate) at pH 7.4 is shown in Tables 1–3. The data in Tables 1 and 3 are derived from experiments in which organisms were exposed to each of the radiolabeled forms of a precursor concomitantly. The data in Table 2 are derived from all experiments in which the radiolabeled form of the precursor was utilized. The mean generation of CO_2 from the 1-carbon of glucose was 2–4 times faster than the mean generation of CO_2 from the 6-carbon (Tables 1–3). In accord with the variability of biochemical data from amastigotes, previously reported [7], there was some variability in the determinations for Table 1. Nevertheless, in each experiment in Tables 1 and 3, generation of CO_2 from the 1-carbon was at least 70% greater than generation from the 6-carbon. Furthermore, if the three experiments of Table 1 are combined with the two experiments of Table 3 (lefthand column), the generation of CO_2 from the 1-carbon was significantly greater than CO_2 generation from the 6-carbon (P 0.03; Student's two-tailed *t*-test, 8 degrees of freedom). With respect to fatty acid oxidation, generation of CO_2 from the 1-carbon of palmitate was twice as fast as generation of CO_2 from the rest of the carbons of palmitate in both of the experiments in Table 1. In contrast, the rate of formation of CO_2 from the 2-carbon of acetate was approximately equal to the rate from the rest of the carbons (i.e. the 1-carbon) of acetate.

The rate of CO_2 formation in organisms pretreated with stibogluconate for 4 hr at pH 7.4, expressed as a percent of the control rate, is shown in Table 2. There was no significant dose-dependent decrease in

Table 1. Rates of metabolism of precursors of CO_2 by *L. mexicana* amastigotes at pH 7.4

Precursor	Precursor concn (mM)	Precursor metabolized to CO_2 (nmoles/10^8 cells/hr)	% Precursor taken up by 2 × 10^7 cells in 0.5 hr
[1-^{14}C]Glucose	0.21	2.0 (0.53–3.0)	3.5 (1.6–5.5)
[6-^{14}C]Glucose	0.21	0.81 (0.24–1.7)	4.1 (1.0–7.2)
[U-^{14}C]Glucose	0.21	0.85 (0.44–1.8)	3.0 (1.2–4.8)
[1-^{14}C]Palmitate	0.21	1.2 (1.2–1.3)	5.4 (5.3–5.5)
[U-^{14}C]Palmitate	0.21	0.62 (0.57–0.67)	6.6 (1.8–11.4)
[2-^{14}C]Acetate	0.41	4.8 (3.0–6.4)	ND
[U-^{14}C]Acetate	0.41	5.8 (2.6–8.7)	ND

L. mexicana amastigotes (5–10 × 10^6) that had not been pretreated with Sb were exposed to radiolabeled precursor at the indicated concentration in a total volume of 150 μl for 30 min; the formation of CO_2 was determined (see Materials and Methods); and the amount of precursor metabolized was calculated. Uptake of precursor into amastigotes was determined in separate experiments in which 2 × 10^7 amastigotes were exposed to precursor for 30 min. The data in this table derive from experiments in which amastigotes were exposed to each of the radiolabeled forms of a precursor concomitantly. Data represent mean (range) of two to four determinations.
ND = not determined.

Table 2. Rates of metabolism of precursors to CO_2 in stibogluconate-treated *L. mexicana* amastigotes at pH 7.4

Precursor	Precursor concn (mM)	Precursor metabolized to CO_2 at [Sb] (μg/ml)			
		0	150	300	500
[1-^{14}C]Glucose	0.21	2.7 ± 1.1*	97 ± 10†	87 ± 13†	86 ± 14†
[6-^{14}C]Glucose	0.21	0.90 ± 0.15	89 ± 11	52 ± 13	31 ± 8
[U-^{14}C]Glucose	0.21	0.85 ± 0.28	90 ± 6	90 ± 12	76 ± 13
[1-^{14}C]Palmitate	0.21	1.3 ± 0.27	74 ± 11	40 ± 11	33 ± 10
[U-^{14}C]Palmitate	0.21	0.60 ± 0.07	49 ± 12	15 ± 6	12 ± 2
[2-^{14}C]Acetate	0.41	11 ± 2.7	98 ± 5.5	93 ± 7	94 ± 6

Amastigotes that had been pretreated for 4 hr with the indicated concentration of antimony in the form of stibogluconate were exposed to ^{14}C-labeled precursor (labeled at the indicated carbon), and the formation of $^{14}CO_2$ was determined.

* Nmoles precursor metabolized to $CO_2/10^8$ cells/hr (mean ± SE: N = 4–7).

† Percent control metabolism (mean ± SE; N = 4–7).

CO_2 formation from 1-carbon of glucose, but there was a significant (P 0.003, 8 degrees of freedom) decrease in metabolism of the 6-carbon of glucose to CO_2 in organisms treated with 500 μg Sb/ml compared to organisms treated with 150 μg Sb/ml. The mean metabolism of the 1-carbon in palmitate, as well as the rest of the carbons, to CO_2 was inhibited by stibogluconate exposure as much as or more than the mean metabolism of the 1-carbon of glucose. Inhibition of CO_2 formation for the 1-carbon of palmitate in organisms exposed to 500 μg Sb/ml was significantly (P 0.05, 6 degrees of freedom) greater than inhibition in organisms exposed to 150 μg Sb/ml. Inhibition of CO_2 formation from the rest of the carbons of palmitate was also decreased significantly (P 0.02, 4 degrees of freedom) at the higher drug concentration. Although the data in Table 2 suggest that the catabolism of carbons 2–16 of palmitate was inhibited to a greater extent than the catabolism of the 1-carbon of palmitate, the values of the mean CO_2 formation in stibogluconate-exposed organisms from [1-^{14}C]palmitate in experiments performed *concomitantly* with [U-^{14}C]palmitate were: 150 μg Sb/ml, 43%; 300 μg Sb/ml, 15%; and 500 μg Sb/ml, 10%. Thus, stibogluconate-induced inhibition of CO_2 formation from the first carbon of palmitate was comparable to the inhibition of oxidation of the other carbons in palmitate. In contrast to the inhibition of [6-^{14}C]glucose and fatty acid catabolism, formation of CO_2 from the 2-carbon in acetate was virtually unaffected by pretreatment of the organisms with stibogluconate.

Mukkada *et al.* [7] recently reported differential rates of glucose catabolism in *Leishmania donovani* amastigotes at pH 4.5, and 7, the former being the putative pH in the phagolysosome in which amastigotes reside. We investigated whether the major features of stibogluconate inhibition of bioenergetic pathways were altered at pH 4.5. As shown in Table 3, the slight inhibition of CO_2 formation from [1-^{14}C]glucose and [2-^{14}C]acetate, and the large inhibition of CO_2 formation from [U-^{14}C]palmitate, seen at pH 7.4 was also seen at pH 4.5. Inhibition of CO_2 formation from [6-^{14}C]glucose at pH 4.5 was greater than the inhibition of CO_2 formation from [1-^{14}C]glucose at pH 4.5, but the difference was not as great as that which occurred at pH 7.4.

Because it has been suggested that organic acids such as succinate are important catabolic metabolites of glucose in amastigotes [8], formation of organic acids in control and stibogluconate-pretreated amastigotes was ascertained. Thin-layer chromatographic

Table 3. Rates of metabolism of precursors to CO_2 in stibogluconate-treated *L. mexicana* amastigotes at pH 7.4 and pH 4.5

Precursor	Precursor concn (mM)	Precursor metabolized to CO_2			
		Without Sb (nmoles/ 10^8 cells/hr)	With Sb (% of control)	Without Sb (nmoles/ 10^8 cells/hr)	With Sb (% of control)
		pH 7.4		pH 4.5	
[1-^{14}C]Glucose	0.21	2.8 (2.7–2.9)	66 (61–70)	0.41 (0.26–0.56)	77 (58–96)
[6-^{14}C]Glucose	0.21	0.66 (0.50–0.81)	22 (12–33)	0.23 (0.21–0.25)	60 (59–60)
[U-^{14}C]Palmitate	0.21	1.4 (1.1–1.7)	23 (10–36)	0.68 (0.68–0.68)	22 (16–28)
[2-^{14}C]Acetate	0.41	5.3 (5.1–5.5)	69 (69–69)	16.0 (15–17)	67 (61–72)

Amastigotes were or were not pretreated with 500 μg Sb/ml in medium at pH 7.4 or pH 4.5 for 4 hr, then exposed to the ^{14}C-labeled precursor, and the formation of CO_2 was determined. Results represent mean (range) of two experiments in which amastigotes were incubated at pH 7.5 or 4.5, concomitantly.

procedures were developed in which succinate was separated from the radiolabeled glucose or fatty acid precursors. On cellulose TLC, succinate was separable from glucose. [1-^{14}C]Glucose was used for these experiments because of its high specific activity relative to [6-^{14}C]glucose. The detection limits of these procedures indicate that formation of succinate from [1-^{14}C]glucose was less than 4% of that of CO_2 from glucose in control (Sb = 0) organisms (Table 4). Since 1-carbon metabolism to CO_2 is about twice that of the 6-carbon this suggests that succinate synthesis from the 6-carbon of glucose was less than 8% of the rate of CO_2 formation. Citric acid cycle intermediates other than succinate were present at a concentration roughly equal to the CO_2 generated. On silica TLC in which succinate was separable from palmitate, formation of succinate from U-labeled palmitate was less than 9% of the rate of CO_2 formation in control parasites. Again, large quantities of metabolites that co-chromatographed with Krebs cycle intermediates were found. In organisms exposed to 150 μg Sb/ml, metabolite formation was 39% of controls, whereas CO_2 formation was 32% of controls. Thus, the decrease of non-succinate organic acids paralleled the decrease in CO_2 from palmitate in stibogluconate-exposed amastigotes.

DISCUSSION

In mammalian bioenergetics, glucose can be metabolized via the hexose monophosphate shunt to produce CO_2 from the 1-carbon of glucose, a 5-carbon sugar, and NADPH. In addition, glucose can be metabolized via part of the glycolytic pathway to the 3-carbon substances phosphoenolpyruvate and then pyruvate, and the latter can be decarboxylated to form acetate. Fatty acids can be metabolized via β-oxidation in which acetate groups are sequentially removed from the carboxyl end of the acid, and NADH and FADH are formed. When both of the carbons in acetate are oxidized to CO_2 in the citric acid cycle, large amounts of NADH and FADH are formed. These then became the substrates of mitochondrial oxidative phosphorylation in which ATP is generated from ADP.

In *L. mexicana* amastigotes, the rate of CO_2 formation from the 1-carbon of glucose was 2–4 times the rate of CO_2 formation from the 6-carbon. Since the 1-carbon is oxidized before carbons 2–6 in the hexose monophosphate shunt, but is equivalent to the 6-carbon in glycolysis, the increased rate of 1-carbon over 6-carbon metabolism to CO_2 has been taken as an approximation of the rate of activity of the hexosemonophosphate shunt. On this basis, the hexosemonophosphate shunt was found to be 1.5–3.0 times faster than glycolysis in *L. mexicana* amastigotes.

As Hart and Coombs reported [8], oxidation of the first carbon of fatty acids (here, palmitic acid) was approximately twice as fast as oxidation of the rest of the molecule. This comparison suggests that half of 1-carbon catabolism to CO_2 occurs in a pathway that removes only that carbon and, therefore, will not generate acetate. The rate of utilization of [U-^{14}C]palmitate to CO_2 may better reflect catabolic processes that generate 2-carbon moieties suitable for entry into the citric acid cycle. In this case, the rate of utilization of fatty acids for processes that are likely to generate NADH was slightly less than the rate of glucose metabolism via glycolytic enzymes.

Mottram and Coombs [2] suggested that glycolysis may proceed only to phosphoenolpyruvate, which would react with CO_2 to form oxaloacetate and then succinate. CO_2 would not be formed from glucose by such pathways. We, however, were unable to detect succinate in these 0.5-hr experiments. This result is consistent with the lack of succinate excretion in the first 24 of 64 hr of *L. mexicana* promastigote cultivation [9]. In the time period employed here, CO_2 formation rather than succinate excretion appears to be the appropriate measure of glucose catabolism.

In our prior work, exposure of amastigotes to 150–500 μg Sb/ml resulted in a dose-dependent decrease of net ATP formation [4]. In the present work, these Sb concentrations cause a dose-dependent decrease

Table 4. Thin-layer chromatographic determination of metabolites of glucose and palmitate in *L. mexicana* amastigotes formed at pH 7.4

Precursor	[Sb]	R_f	Standards	Metabolite* (nmoles/10^8 cells/hr)	CO_2 (nmoles/10^8 cells/hr)
[1-^{14}C]Glucose†	0	0.0–0.21	Organic acids	1.20	1.1
		0.57–0.64	Succinate/Lactate	≤0.04	
[U-^{14}C]Palmitate‡	0	0.0–0.07	Organic acids	1.88	0.23
		0.14–0.24	Succinate/Lactate	≤0.02	
	150	0.0–0.07	Organic acids	0.73 (39% of 1.88)	0.07 (32% of 0.23)
		0.14–0.21	Succinate/Lactate	None	None

Amastigotes treated with 0 or 150 μg Sb/ml at pH 7.4 were exposed to the radiolabeled precursor for 30 min and then extracted with trichloroacetic acid. Portions of the TCA-extract were chromatographed with cold precursor, organic acids, succinate, and lactate as external standards. Chromatographic regions corresponding to the external standards were cut out, and scintillation was counted. Concomitant formation of CO_2 from the precursor was also determined.

* Calculation assumes that 1 nmole metabolite is formed from 1 nmole precursor.

† PEI-cellulose chromatography. The co-chromatographing organic acids were pyruvic, acetic, α-ketoglutaric, and oxaloacetic. The R_f for glucose was 0.28–0.43.

‡ Silica gel-IB2F chromatography. The co-chromatographing organic acids were pyruvic, α-ketoglutaric, malic, and oxaloacetic. The R_f for palmitate was 0.80–0.87.

in formation of CO_2 via glycolytic enzymes and fatty acid β-oxidation. The lack of stibogluconate inhibition of the acetate $\rightarrow CO_2$ pathway indicates that stibogluconate treatment did not inhibit utilization of acetate generated by glycolytic enzymes or β-oxidation. Thus, inhibition of CO_2 formation from glucose and fatty acid resulted from inhibition of a process between the uptake of the precursors and the generation of acetate.

The fact that utilization of glucose in the hexose-monophosphate shunt was relatively unaffected by stibogluconate indicates that stibogluconate did not inhibit glucose transport into the cell or phosphorylation of glucose to glucose-6-phosphate. Inhibition of formation of CO_2 from [6-^{14}C]glucose may be due to inhibition of the generation of acetate from glucose-6-phosphate, that is, due to inhibition of glycolytic enzymes themselves. Bueding and coworkers demonstrated in the 1950s that 30–300 μg/ml of trivalent antimonials inhibit schistosomal phosphofructokinase to a greater extent than the mammalian enzyme [10, 11], and one of the sites of stibogluconate activity against amastigotes may be inhibition of leishmanial phosphofructokinase or other glycolytic enzymes. Alternatively, the fact that *Leishmania* promastigotes and, presumably, amastigotes segregate their glycolytic enzymes into organelles (glycosomes) [12] suggests that inhibition of glycosome structure or function by stibogluconate may result in inhibition of glycolytic enzymes.

The manner in which fatty acids are presented to β-oxidative enzymes has not been clarified for *Leishmania*, but the fact that oxidation of 1-carbon of palmitate was inhibited to approximately the same extent as oxidation of the rest of palmitate suggests that some basic aspect of fatty acid transport, rather than β-oxidation *per se*, may be inhibited by stibogluconate.

The demonstration that two of three major enzymatic pathways leading to energy generation in amastigotes are inhibited by stibogluconate suggests that these mechanisms may significantly account for the apparent inhibition of the ADP to ATP reaction previously reported in stibogluconate-exposed organisms.

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REFERENCES

1. E. A. Steck, *The Chemotherapy of Protozoan Disease*, p. 7–3. U.S. Government Printing Office, Washington, DC (1972).
2. J. C. Mottram and G. H. Coombs, *Expl. Parasit.* **59**, 151 (1985).
3. J. D. Berman, G. G. Holz and D. H. Beach, *Molec. Biochem. Parasit.* **12**, 1 (1984).
4. J. D. Berman, D. Waddell and B. D. Hanson, *Antimicrob. Agents Chemother.* **27**, 916 (1985).
5. K. P. Chang, *Science* **209**, 1240 (1980).
6. J. E. Decker, J. R. Schrot and G. V. Levin, *J. Protozool.* **24**, 463 (1974).
7. A. J. Mukkada, J. C. Meade, T. A. Glaser and P. F. Bonventre, *Science* **229**, 1099 (1985).
8. D. T. Hart and G. H. Coombs, *Expl. Parasit.* **54**, 397 (1982).
9. J. J. Cazzulo, B. M. F. de Cazzulo, J. C. Engel and J. J. B. Cannata, *Molec. Biochem. Parasit.* **16**, 329 (1985).
10. T. E. Mansour and E. Bueding, *Br. J. Pharmac. Chemother.* **9**, 459 (1954).
11. E. Bueding and J. M. Mansour, *Br. J. Pharmac. Chemother.* **12**, 159 (1957).
12. D. T. Hart and F. R. Opperdoes, *Molec. Biochem. Parasit.* **13**, 159 (1984).