ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1993, p. 1842–1846
0066-4804/93/091842-05$02.00/0

Vol. 37, No. 9

Antileishmanial Activity of Sodium Stibogluconate Fractions

WILLIAM L. ROBERTS AND PETRIE M. RAINEY*

Department of Laboratory Medicine, Yale University, New Haven, Connecticut 06510

Received 25 March 1993/Returned for modification 20 May 1993/Accepted 22 June 1993

Sodium stibogluconate, a pentavalent antimony derivative produced by the reaction of stibonic and gluconic acids, is the drug of choice for the treatment of leishmaniasis. It has been reported to be a complex mixture rather than a single compound. We separated sodium stibogluconate into 12 fractions by anion-exchange chromatography. One fraction accounted for virtually all the leishmanicidal activity of the fractionated material against *Leishmania panamensis* promastigotes, with a 50% inhibitory concentration (IC_{50}) of 12 μg of Sb per ml; that of unfractionated sodium stibogluconate was 154 μg of Sb per ml. Further analysis of this active fraction revealed that a major component was *m*-chlorocresol, which had been included in the sodium stibogluconate formulation as a preservative. The IC_{50} of pure *m*-chlorocresol was 1.6 μg/ml, a concentration equivalent to that present in unfractionated sodium stibogluconate at a concentration of 160 μg of Sb per ml. After ether extraction to remove *m*-chlorocresol, the IC_{50} of sodium stibogluconate was >4,000 μg of Sb per ml. In contrast, when *L. panamensis* amastigotes were grown in macrophages, the IC_{50} of ether-extracted sodium stibogluconate was 10.3 μg of Sb per ml. The 12 fractions of ether-extracted sodium stibogluconate obtained by anion-exchange chromatography had IC_{50}s of 10.1 to 15.4 μg of Sb per ml. We conclude that preservative-free sodium stibogluconate has little activity against *L. panamensis* promastigotes but is highly active against *L. panamensis* amastigotes in macrophages. This activity is associated with multiple chemical species.

Pentavalent antimony complexed with carbohydrate in the form of sodium stibogluconate has been the treatment of choice for the various forms of leishmaniasis for nearly 50 years. The chemical composition of this agent remains unknown, but early studies on pentavalent antimonial agents suggested that the state of molecular aggregation was fundamental to drug action and that proper aging was necessary to obtain substances having maximum efficacy and acceptable toxicity (13 and references cited therein). Some lots of sodium stibogluconate have proven to be clinically ineffective (8). Recent evidence indicated that sodium stibogluconate is a complex mixture of components with apparent molecular masses ranging from 100 to 4,000 Da (3).

The majority of drugs work through specific interactions with proteins, usually an enzyme or a receptor. In such instances, activity is highly dependent on molecular structure (function follows form). If sodium stibogluconate follows this paradigm, it can be hypothesized that the majority of antileishmanial activity resides in a single component of the mixture that has the proper structure for interaction with the drug target. Should this hypothesis be true, a preparation of the purified component should exhibit enhanced efficacy and also may have diminished toxicity. For testing this hypothesis, sodium stibogluconate was fractionated by anion-exchange chromatography, and the 50% inhibitory concentration (IC_{50}) of each fraction was determined with *Leishmania panamensis* axenic promastigotes and with intracellular amastigotes grown in mouse macrophage cell line J774G8.

MATERIALS AND METHODS

All chemicals were of reagent grade or better. Potassium antimonyltartrate (99.95% purity) and *m*-chlorocresol (4-chloro-3-methylphenol) were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Sodium stibogluconate (Pentostam) was obtained from the Centers for Disease Control (Atlanta, Ga.).

Ether-extracted sodium stibogluconate was prepared by extracting 4 ml of sodium stibogluconate (100 mg of Sb per ml) with two 10-ml portions of diethyl ether and discarding the organic phases. Residual ether was removed from the aqueous phase under a stream of air for 15 min at 40°C. Anion-exchange chromatography of sodium stibogluconate and ether-extracted sodium stibogluconate (10 mg of Sb each) was performed by use of a Mem-Sep DEAE cartridge (Millipore Corp., Bedford, Mass.) with a linear gradient of sodium chloride (0 to 0.5 M) in sodium phosphate buffer (10 mM, pH 7.0) over 30 min at a flow rate of 1 ml/min. The final sodium chloride concentration was maintained for 10 min prior to reequilibration in sodium chloride-free buffer. The A_{230} was monitored. Twelve fractions of approximately 3 ml each were collected on the basis of apparent peaks seen on the UV trace (Fig. 1). Larger peaks were collected as multiple fractions. The antimony concentrations in the fractions were quantified by graphite furnace atomic absorption spectroscopy as previously described (11). The recovery of applied antimony was 80%. Fractions were concentrated for further experiments by use of a Speed-Vac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.). UV spectra were recorded on a UV-260 spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, Md.).

Promastigote growth inhibition studies were done with *L. panamensis* WR120 (World Health Organization designation MHOM/PA/74/WR120) grown at 22°C in 5 ml of UM-55 (10) in 25-cm^2 tissue culture flasks. Promastigotes in the log phase were seeded at a density of 1×10^6 to 2×10^6/ml, drug treatment was initiated, and daily counts were made electronically with a model ZF counter (Coulter Electronics, Inc., Hialeah, Fla.) for 3 days. Growth rate constants were calculated from a plot of log parasite number versus time. The drug concentration producing a 50% reduction in the growth rate constant (IC_{50}) was estimated from least-squares linear regression of the growth rate constant versus concentration for sodium stibogluconate, its fractions, and *m*-chlo-

* Corresponding author.

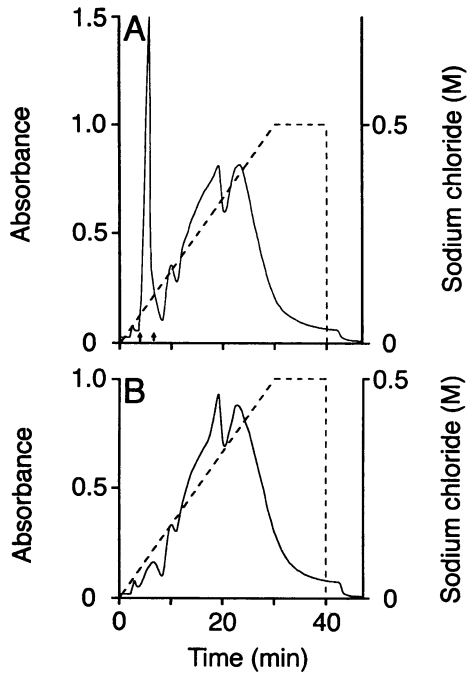

FIG. 1. Fractionation of sodium stibogluconate (A) and ether-extracted sodium stibogluconate (B) by anion-exchange chromatography. Chromatography was performed by use of a DEAE cartridge with a linear gradient of sodium chloride (– – –), and the A_{230} was monitored (——) (see Materials and Methods). Fraction 2 in panel A is bracketed by two arrows.

rocresol and from a log concentration plot for potassium antimonyltartrate and ether-extracted sodium stibogluconate.

Promastigote growth inhibition by sodium stibogluconate, *m*-chlorocresol, ether-extracted sodium stibogluconate, and potassium antimonyltartrate was also quantified on the basis of [^{3}H]uracil incorporation. Log-phase promastigotes (1×10^6 to 2×10^6/ml) were grown in 200 μl of UM-55 containing serial dilutions of these drugs in triplicate. After 2 days of log-phase growth, 1 μCi of [^{3}H]uracil (Moravek Biochemicals Inc., Brea, Calif.) was added, and after overnight incubation, the parasites were harvested onto filter mats with a Combi harvester (Skatron Inc., Sterling, Va.). The mats were sealed in plastic bags containing 10 ml of Beta Plate Scint cocktail (Wallac Inc., Gaithersburg, Md.), and counting was done with a model 1205 Beta Plate counter (Wallac). Counts per minute in drug-treated cultures were expressed as a percentage of those in untreated controls. For each experiment, the concentration of drug producing a 50% reduction in the control counts per minute (IC_{50}) was estimated from a log concentration plot.

Amastigote growth inhibition studies were done with mouse macrophage cell line J774G8 (5), which was cultivated at 35°C in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (complete RPMI). Macrophages were grown to confluence in 100 μl of complete RPMI per well in 96-well, flat-bottom tissue culture plates. Suspensions of *L. panamensis* WR120 promastigotes grown in UM-55 to the late log phase (5×10^7 to 10×10^7/ml) were diluted with complete RPMI to a density of 2×10^7/ml. An aliquot (100 μl) containing 2×10^6 promastigotes was added to each well of the plate. After overnight incubation, at the end of which all promastigotes were observed by inverted microscopy to have been phagocytosed, the old medium was aspirated and serial dilutions of sodium stibogluconate and its fractions in fresh complete RPMI (200 μl) were added in triplicate. After 5 days, leishmanial growth was assessed by the addition to each well of 1 μCi of [^{3}H]uracil in 50 μl of RPMI 1640 (2). After overnight incubation, the cells were harvested and counting was done as described above. Net counts per minute in drug-treated infected cultures (counts per minute in treated infected cultures minus counts per minute in treated uninfected controls) were expressed as a percentage of those in untreated infected controls. For each experiment, the concentration of drug producing a 50% reduction in the net counts per minute in untreated infected controls (IC_{50}) was estimated from a log concentration plot.

TABLE 1. Activities of antileishmanial agents against *L. panamensis* promastigotes

Compound	Mean IC_{50}[a]	SEM	No. of determinations
Unfractionated sodium stibogluconate	154	20	3
Fraction 1[b]	>200	ND[c]	2
Fraction 2[b]	12	3	3
Fractions 3–12[b]	>200	ND	2
Ether-extracted sodium stibogluconate	>4,000	ND	4
m-Chlorocresol	1.6[d]	0.2	3
Potassium antimonyl-tartrate	2.1	0.3	3

[a] Expressed as micrograms of Sb per milliliter of culture medium unless otherwise indicated.
[b] Sodium stibogluconate was fractionated by anion-exchange chromatography and fractions were collected as described in Materials and Methods.
[c] ND, not determined.
[d] Expressed as total micrograms per milliliter.

RESULTS

Sodium stibogluconate was subjected to anion-exchange chromatography by use of gradient elution with sodium chloride (Fig. 1A). Twelve fractions of approximately 3 ml each were collected over a 35-min period, with an 80% recovery of applied antimony. The IC_{50} of each fraction for *L. panamensis* promastigotes was determined (Table 1). Fraction 2, with a retention time of 4 to 7 min, had an IC_{50} of 12 μg of Sb per ml; the IC_{50} of unfractionated sodium stibogluconate was 154 μg of Sb per ml. The other 11 fractions all had IC_{50}s that were much higher than 200 μg of Sb per ml. UV spectra of unfractionated sodium stibogluconate (Fig. 2A) and fraction 2 (Fig. 2B) both exhibited an unexpected absorbance peak in the 270- to 290-nm region. Absorbance peaks in this region can be associated with conjugated aromatic systems (12). The liquid formulation of sodium stibogluconate used in these investigations was preserved with *m*-chlorocresol, present at a concentration of 0.1% (1 mg/ml). The UV spectrum of pure *m*-chlorocresol at a concentration equivalent to that estimated to be present in the sodium stibogluconate in Fig. 2A is shown in Fig. 2C. The A_{280}s were virtually identical in these two spectra, confirming that similar concentrations of *m*-chlorocresol were present in both samples. Ether extraction of sodium stibogluconate yielded material devoid of the aromatic absorbance peak (Fig. 2D), indicating that *m*-chlorocresol

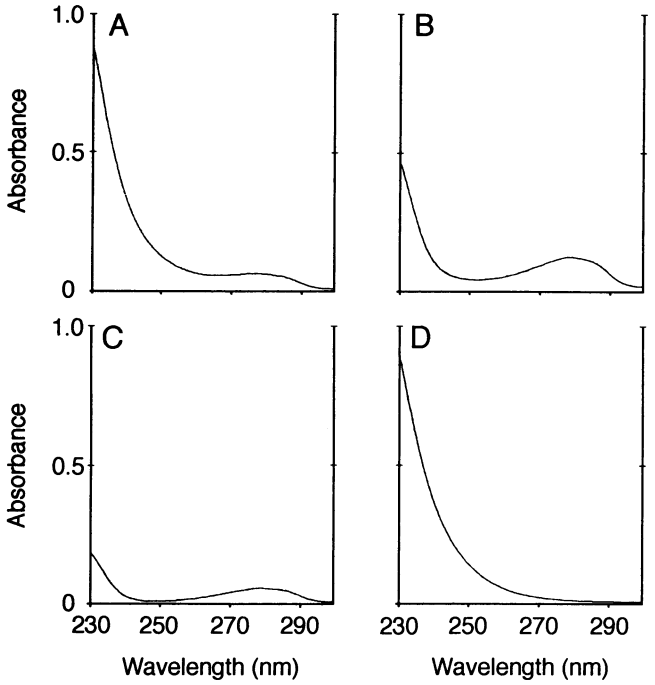

FIG. 2. UV spectra of sodium stibogluconate and fractions. (A) Unfractionated sodium stibogluconate (1 mg of Sb per ml). (B) Fraction 2 from the DEAE cartridge (0.15 mg of Sb per ml). (C) *m*-Chlorocresol (0.01 mg/ml) in water. (D) Ether-extracted sodium stibogluconate (1 mg of Sb per ml).

could be readily removed by this procedure. We further confirmed that fraction 2 contained *m*-chlorocresol by subjecting ether-extracted sodium stibogluconate to anion-exchange chromatography; the results are shown in Fig. 1B. The UV absorbance associated with fraction 2 was markedly reduced by ether extraction, consistent with the removal of *m*-chlorocresol.

The antileishmanial properties of *m*-chlorocresol and ether-extracted sodium stibogluconate were investigated with promastigotes. Potassium antimonyltartrate, a trivalent antimony compound, was included as a positive control. The results are summarized in Table 1. The IC_{50} of *m*-chlorocresol was 1.6 μg/ml, a concentration essentially equivalent to that present in unfractionated sodium stibogluconate at the latter's IC_{50} of 154 μg of Sb per ml. Furthermore, the IC_{50} of ether-extracted sodium stibogluconate was >4,000 μg of Sb per ml. These results indicate that most if not all of the activity of unfractionated sodium stibogluconate against *L. panamensis* promastigotes was produced by the preservative, *m*-chlorocresol. Potassium antimonyltartrate was inhibitory to promastigote growth, with an IC_{50} of 2.1 μg/ml, indicating that WR120 promastigotes are susceptible to a trivalent antimony compound.

The effects of sodium stibogluconate and its fractions on *L. panamensis* amastigotes grown in mouse macrophage cell line J774G8 were quite different from those observed for promastigotes (Table 2). The IC_{50} of ether-extracted sodium stibogluconate for intracellular amastigotes was 10.3 μg of Sb per ml, at least 2 orders of magnitude lower than that observed for promastigotes. Fractions 1 to 12 from anion-exchange chromatography all had similar IC_{50}s, which ranged from 10.1 to 15.4 μg of Sb per ml. The IC_{50} of the nonextracted sodium stibogluconate control was 12.1 μg of Sb per ml. In contrast, *m*-chlorocresol showed no inhibition at concentrations of up to 0.32 μg/ml, which corresponded to its concentration in nonextracted sodium stibogluconate at a concentration of 32 μg of Sb per ml. Higher concentrations of *m*-chlorocresol produced toxicity in the J774G8 cells (macrophage toxicity was not observed at concentrations of preservative-free sodium stibogluconate of up to 256 μg of Sb per ml or of potassium antimonyltartrate of up to 1.6 μg of Sb per ml). The IC_{50} of potassium antimonyltartrate in this system was 12 ng of Sb per ml, again at least 2 orders of magnitude lower than that measured for promastigotes.

TABLE 2. Activities of ether-extracted sodium stibogluconate components against *L. panamensis* amastigotes in J774G8 cells

Compound	IC_{50}[a]	SEM	No. of determinations
Ether-extracted sodium stibogluconate	10.3	1.2	7
Fraction 1[b]	12.2	0.1	2
Fraction 2[b]	11.5	0.5	2
Fraction 3[b]	12.4	0.1	2
Fraction 4[b]	15.0	0.2	2
Fraction 5[b]	12.9	2.0	2
Fraction 6[b]	12.7	1.9	2
Fraction 7[b]	14.0	2.5	2
Fraction 8[b]	15.4	2.8	2
Fraction 9[b]	14.0	2.6	2
Fraction 10[b]	12.6	3.5	2
Fraction 11[b]	12.0	0.8	2
Fraction 12[b]	10.1	0.4	2
Nonextracted sodium stibogluconate (control)	12.1	2.9	2
m-Chlorocresol[c]	>0.32	ND[d]	2
Potassium antimonyl-tartrate	0.012	0.002	5

[a] See Table 1, footnote *a*.
[b] Ether-extracted sodium stibogluconate was fractionated by anion-exchange chromatography and fractions were collected as described in Materials and Methods.
[c] See Table 1, footnote *d*.
[d] ND, not determined.

For confirmation that parasite number and uracil incorporation were roughly equivalent measures of leishmanial growth, promastigote growth inhibition was also quantified on the basis of [^{3}H]uracil incorporation after 2 days of log-phase growth in the presence of drug. The IC_{50}s were 187 μg of Sb per ml for sodium stibogluconate, 2.2 μg/ml for *m*-chlorocresol, >4,000 μg of Sb per ml for ether-extracted sodium stibogluconate, and 1.4 μg of Sb per ml for potassium antimonyltartrate, all of which were comparable to those found by electronic cell counting (Table 1).

DISCUSSION

Sodium stibogluconate was previously shown to be a complex mixture. Our goal was to isolate a fraction with enhanced antileishmanial activity. Anion-exchange chromatography provided partial resolution of the mixture, and 12 fractions were evaluated for activity in two in vitro systems. The first system used axenic *L. panamensis* promastigotes and had the advantage of simplicity. Axenically grown promastigotes are the most widely used models for the study of antileishmanial drugs. The second system used amastigotes of the same strain grown in macrophage cell line J774G8, a model that more closely resembles the situation in a treated host.

In the promastigote system, the activity of the liquid

sodium stibogluconate formulation was primarily derived from the preservative, *m*-chlorocresol. Preservative-free sodium stibogluconate was essentially inactive against *L. panamensis* promastigotes, even though potassium antimonyltartrate, a trivalent antimony control, showed activity, with an IC_{50} of 2.1 μg of Sb per ml. The susceptibility of these promastigotes to *m*-chlorocresol, coupled with their relative resistance to preservative-free sodium stibogluconate, indicates a potential problem in the use of promastigotes as a model system. It also brings into question earlier results obtained for promastigotes with sodium stibogluconate. Two forms of sodium stibogluconate generally have been available; a preserved liquid formulation and a dry powder without a preservative. For many previous reports in the literature, it is difficult to determine which form of sodium stibogluconate was used. *m*-Chlorocresol may inhibit oxidative phosphorylation (4). This possibility raises questions about earlier results suggesting that the inhibition of energy metabolism was the mechanism of sodium stibogluconate action. If previous studies used sodium stibogluconate containing *m*-chlorocresol, the inhibition of energy metabolism observed could have been an artifact from the preservative. In retrospect, it is not surprising that a preservative, added to prevent bacterial growth, should prove toxic for protozoa, including *Leishmania* spp. Pharmaceutical preparations often contain additives in addition to the active ingredient. If an additive has pharmacologic activity, the use of such preparations instead of pure materials may result in artifacts.

Nonextracted sodium stibogluconate had an IC_{50} of 12.1 μg of Sb per ml for WR120 amastigotes in J774G8 macrophages. This concentration corresponds to an *m*-chlorocresol concentration of 0.12 μg/ml, which is well below the IC_{50} of pure *m*-chlorocresol in this system. Ether-extracted sodium stibogluconate and its 12 fractions had comparable activities, with IC_{50}s of 10 to 15 μg of Sb per ml in the amastigote-J774G8 macrophage system. These IC_{50}s correspond to achievable levels in serum (6) and are much lower than the IC_{50} of ether-extracted sodium stibogluconate for promastigotes. Since all the pentavalent antimony components in sodium stibogluconate are equipotent, their exact chemical structures seem unimportant for antileishmanial activity. Although all fractions showed equivalent activities in vitro, it remains to be proven that all fractions are equipotent in whole-animal experiments. For example, one fraction could have a longer half-life and hence be responsible for most of the in vivo activity of a drug. This possibility may explain the observed clinical ineffectiveness of some sodium stibogluconate lots (8).

Potassium antimonyltartrate was nearly 3 orders of magnitude more potent than sodium stibogluconate against amastigotes in cell line J774G8 (IC_{50}s, 12 ng of Sb per ml versus 10.3 μg of Sb per ml). This finding, combined with the difference in potency of ether-extracted sodium stibogluconate against intracellular versus extracellular leishmaniae, suggests a possible role for macrophages in the action of antimonial compounds. It has been demonstrated in vivo that pentavalent antimony is reduced to trivalent antimony (7). This conversion could occur in macrophages and contribute to the increased activity of pentavalent antimonial agents against intracellular parasites. Thus, sodium stibogluconate may serve as a passive system for delivering antimony to the reticuloendothelial system, in which active antimony species, possibly trivalent in nature, are generated in situ.

Potassium antimonyltartrate was more than 2 orders of magnitude more potent against intracellular amastigotes than against promastigotes of the same strain (IC_{50}s, 12 ng of Sb per ml versus 2.1 μg of Sb per ml), indicating that factors other than the generation of trivalent species are also operative in the modification of drug action against intracellular parasites. This result is unlikely to be explained by a higher inherent susceptibility of amastigotes than of promastigotes to trivalent antimony, since previous work with *L. pifanoi* indicated that the opposite was true: the IC_{50}s of potassium antimonyltartrate were 20 μg of Sb per ml for axenic amastigotes but only 1 to 2 μg of Sb per ml for promastigotes (11). Several other possibilities exist. First, macrophages could concentrate antimony from the extracellular medium so that the antimony concentration in parasitophorous vacuoles is substantially higher than that in the medium. Second, amastigotes could exhibit increased permeability to trivalent antimony when resident in parasitophorous vacuoles and accumulate antimony more rapidly. Third, macrophages could metabolize potassium antimonyltartrate to a more toxic species. Finally, other mechanisms of macrophage parasite toxicity might be synergistic with the action of antimonial agents. For example, the activation of macrophages with gamma interferon resulted in a decreased requirement for antimony in model systems (9), while treatment with gamma interferon and pentavalent antimony produced cures in patients unresponsive to treatment with pentavalent antimony alone (1). Regardless of the mechanism of enhanced toxicity in macrophages, further work to elucidate the mechanism of action of antimonial agents may require a system that uses macrophages to adequately simulate the conditions present in vivo.

ACKNOWLEDGMENTS

We thank J. D. Berman for careful review of the manuscript. The J774G8 cells and *L. panamensis* WR120 promastigotes were graciously provided by Diane McMahon-Pratt.

This work was supported in part by the National Institutes of Health (grant AI-27382 to P.M.R.), by the Merck/American Federation for Clinical Research Foundation (postdoctoral fellowship to W.L.R.), and by the AB Foundation for Medical Research (protocol 92-02 to P.M.R.).

REFERENCES

1. **Badaro, R., E. Falcoff, F. S. Badaro, et al.** 1990. Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma. N. Engl. J. Med. **322**:16–21.
2. **Berman, J. D., and J. V. Gallalee.** 1985. Semiautomated assessment of in vitro activity of potential antileishmanial drugs. Antimicrob. Agents Chemother. **28**:723–726.
3. **Berman, J. D., and M. Grogl.** 1988. *Leishmania mexicana*: chemistry and biochemistry of sodium stibogluconate (Pentostam). Exp. Parasitol. **67**:96–103.
4. **Biggs, D. F.** 1965. Experiments on the mechanism of action of chlorocresol and caffeine. Br. J. Pharmacol. **24**:510–518.
5. **Chang, K.-P.** 1980. Human cutaneous leishmania in a mouse macrophage line: propagation and isolation of intracellular parasites. Science **209**:1240–1242.
6. **Chulay, J. D., L. Fleckenstein, and D. H. Smith.** 1988. Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. Trans. R. Soc. Trop. Med. Hyg. **82**:69–72.
7. **Goodwin, L. G., and J. E. Page.** 1943. A study of the excretion of organic antimonials using a polarographic procedure. Biochem. J. **22**:236–240.
8. **Jackson, J. E., J. D. Tally, W. Y. Ellis, et al.** 1990. Quantitative in vitro drug potency and drug susceptibility evaluation of *Leishmania* spp. from patients unresponsive to pentavalent antimony therapy. Am. J. Trop. Med. Hyg. **43**:464–480.
9. **Murray, H. W., J. D. Berman, and S. D. Wright.** 1988. Immu-

notherapy for intracellular *Leishmania donovani* infection: γ interferon plus pentavalent antimony. J. Infect. Dis. **157:**973–978.

10. **Rainey, P. M., T. W. Spithill, D. McMahon-Pratt, and A. A. Pan.** 1991. Biochemical and molecular characterization of *Leishmania pifanoi* amastigotes in continuous axenic culture. Mol. Biochem. Parasitol. **49:**111–118.
11. **Roberts, W. L., and P. M. Rainey.** 1993. Antimony quantification in *Leishmania* by electrothermal atomic absorption spectroscopy. Anal. Biochem. **211:**1–6.
12. **Scott, A. I.** 1964. Interpretation of the ultraviolet spectra of natural products, p. 89–134. Pergamon Press, Inc., Tarrytown, N.Y.
13. **Steck, E. A.** 1974. The leishmaniases. Prog. Drug Res. **18:**289–351.