Trypanocidal Activity of Antitumor Antibiotics and Other Metabolic Inhibitors

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A microtest has been devised for the rapid preliminary assay in vitro of the effect of over 100 drugs and inhibitors on African trypanosomes (Trypanosoma brucei and T. rhodesiense). Parasite motility and infectivity for mice are indexes, respectively, of respiration and glycolysis and of cell division; trypanocidal titers based on these indexes can show primary metabolic areas of drug attack. Various specific inhibitors have also been tested to detect metabolic sites which might be therapeutically vulnerable. RNA synthesis inhibitors are highly active (adenine nucleosides, daunorubicin, doxorubicin, chromomycin, actinomycin D, mitomycin C); the activity of the nucleoside cordycepin was increased in vitro and in vivo by an adenosine deaminase inhibitor. In view of the polyanionic nature of the trypanocide suramin, a series of polyanions was tested; several showed activity but only poly-D-glutamic acid was active in vivo. Among various miscellaneous inhibitors, quercetin, disulfiram, and the Ca-complexing agents arsenazo I and III showed marked activity, the latter exclusively on the arsenical-resistant T. brucei.

The implications of these results for combination chemotherapy and depot prophylaxis (with polyanions) are indicated.

New drugs are urgently required for African human and animal trypanosomiasis, especially the latter (52, 53). In the absence of new products from large-scale commercial drug screening, academic laboratory investigation may provide useful leads by concentrating on aspects of trypanosome metabolism which may be vulnerable to chemotherapeutic attack. Susceptible loci exist because the parasitic mode of pathogenic trypanosomes has induced dependence on host metabolites and loss of major components of the respiratory, glycolytic, and biosynthetic functions normally found in mammalian host cells.

These metabolic lesions and adaptations indicate vulnerable points of selective trypanocidal drug attack. A high aerobic glycolysis rate is associated with extreme sensitivity to phenylarsenoxides, and absence of cytochromes has given rise to reliance on a parasite-specific \( \alpha \)-glycerophosphate oxidase-mediated respiration which is highly sensitive to inhibition by suramin (11) and by aromatic hydroxamates (10, 31); recent analyses of the function of this system (5, 12) have led to the demonstration that trypanosomes can be killed by using salicylhydroxamate and glyceral for simultaneous selective blockade of aerobic and anaerobic glycolysis. Defective purine biosynthesis is also open to attack by adenine nucleosides such as puromycin and cordycepin (52).

A number of drugs and inhibitors with specific action sites in other kinds of cell have been used to look for susceptible loci in trypanosomes (cf. 50), and the search has been extended using certain antitumor antibiotics whose loci of action have recently been specified with increasing precision; any trypanocidal activity in these compounds has been assumed to indicate that the specific action loci, as determined in other kinds of cell, are also present in trypanosomes. Several are especially active on macromolecular synthesis, so that rapidly dividing cells such as proliferative tumors tend to be selectively inhibited. Pathogenic trypanosomes, malaria parasites, and schistosomes are also likely to be susceptible to such agents, and particularly to inhibitors of nucleic acid synthesis, since all three parasites appear to be unable to make their own purine bases (25, 48, 52).

Trypanocidal activity tests of a number of representative compounds of this kind have revealed unsuspected high activity in several, especially among RNA synthesis inhibitors, as indicated in a preliminary report (56).

MATERIALS AND METHODS

Trypanosomes (Trypanosoma brucei and T. rhodesiense) were obtained from heparinized blood taken by sterile cardiac puncture from heavily infected mice. Both trypanosome strains, which were uniformly fatal.
for mice in 3 to 4 days, had been maintained by syringe passage in mice and by freezer storage for at least 10 years in the case of *T. brucei* and 30 years in the case of *T. rhodesiense* (59). The provenance of the former strain is unknown, and it will be referred to here as *T. brucei* (PU). The *T. rhodesiense* strain consists exclusively of long, thin forms, whereas *T. brucei* (PU) is more pleomorphic, consisting predominantly of "intermediate" forms. A volume of 1 to 2 drops of infected blood was added to 5 or 10 ml of equal parts of inactivated calf serum and Krebs Ringer-0.2% (wt/vol) glucose medium, and the parasite density was adjusted to $10^5$ per ml after a hemacytometer count. Medium samples of 0.18 ml were distributed in plastic Microtest II plates (Falcon Plastics); a flat-well plate was used for direct examination of the parasites in the wells with an inverted microscope (Wild M40, ocular ×10, objective ×40). In a few cases, where deeply colored test solutions prevented use of the inverted microscope, samples of well contents were examined as cover-slip preparations in a conventional microscope. Test compounds were serially diluted (×10) using an automatic microliter pipette (Gilson Pipetman; Oxford Laboratory Sampler); the advantages of this technique have been convincingly demonstrated (26). After adding trypanosomal suspensions (20 μl) (i.e., to a final density of $10^5$ per ml) and incubating for 4 h at 37°C, trypanosome numbers and motility were assessed, and infectivity was checked by intraperitoneal injection of the well contents into mice (one mouse per well), which were subsequently examined daily for development of parasitemia. Injection of untreated control suspensions was invariably fatal within 4 days. Total abolition of infectivity was concluded if no parasites were detectable over a period of 30 days, blood being considered negative if no trypanosomes were seen in 30 microscopic fields (ocular ×5, objective ×40) in a cover-slip preparation of a drop of tail blood. Negative parasitemia followed by relapse was considered as a temporary abolition of infectivity.

Trypanocidal titer is generally expressed as log₁₀ M⁻¹ (10) and represents the lowest concentration abolishing motility or infectivity; titers less than 3 (i.e., mM) were not considered to be physiologically significant. Concordance among replicates (up to four) was sufficiently good to justify assessment on the basis of single or duplicate assays.

For in vivo drug tests, infected Parkes mice (20 to 25 g) were treated intraperitoneally (0.5 ml/20 g) with aqueous solutions. Subsequent parasitemia was followed daily by microscopic examination of tail blood (objective ×40, ocular ×8) in a cover-slip preparation. Absence of trypanosomes in 30 microscope fields for at least 30 days was taken as a criterion of cure.

Test compounds were of commercial origin, except for the following, which were generous gifts from the sources indicated. ICRF 159 and ICRF 162 from A. M. Creighton, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London; tubercidin, decocine, pascofuramine, and 2-fluorodesoxo from A. Bloch, Roswell Park Memorial Institute, Buffalo, N.Y.; cordycepin N-oxide from S. Frederikaen, Universitas Biokemiisk Institut B, Copenhagen, Denmark; formycin A from D. K. Robins, University of Utah; nucleocidin from A. T. Menzie, Lederle Laboratories, London; distamycin from D. H. Williamson, National Institute for Medical Research, London; erythro-9-(2-hydroxy-3-nonyl)adenine from Gertrude B. Elion, Wellcome Research Laboratories, Research Triangle Park, N.C.; camptothecin and hadacidin from H. B. Wood, National Cancer Institute; nogalamycin from Gary L. Neil, The Upjohn Co., Kalamazoo, Mich.; polyethylene sulfonate from Upjohn Ltd., Crawley, Sussex, England; doxorubicin (adriamycin) from Farmitalia, Milan, Italy; nigericin from Lilly Research Centre Ltd., Widdlesham, Surrey; cordycepin, prepared in bulk, from the Microbial Research Establishment, Porton Down, Wilts; Cibacron blue 3G-6A and Cibacron brilliant blue BR-P from T. Green, Ciba-Geigy (U.K.) Ltd., Manchester; metranidazole from J. A. McFadzean, May and Baker Ltd., Dagenham, Essex; and Bromo-X-537A from P. G. Philpott, Roche Products Ltd., Welwyn Garden City, Herts, England.

**RESULTS**

In the in vitro assay used here, trypanosome motility correlates well with respiration and glycolysis (54), and infectivity for mice is a useful index of cell division (macromolecular synthesis), since bloodstream forms of African trypanosomes cannot be kept in axenic culture. The two kinds of activity titer are thus a useful preliminary guide to whether an inhibitor acts on energy-producing reactions, on macromolecular synthesis, or on both. Inactivity caused by impermeability or by necessity for conversion in vivo to an active form cannot be distinguished in this test, and these important considerations have been largely ignored in the interests of primary activity data accumulation.

As a preliminary check, the drug reactivity of the two trypanosome strains was compared. Table 1 shows that, except for oxophenarsine and acriflavine, all the standard trypanocides were equally active in vitro against both strains. In *T. brucei* (PU), both motility and infectivity were

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>T. brucei</em> (PU)</th>
<th><em>T. rhodesiense</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>Infectivity</td>
<td>Motility</td>
</tr>
<tr>
<td>Oxophenarsine</td>
<td>5 (4)</td>
<td>8 (5)</td>
</tr>
<tr>
<td>Acriflavine</td>
<td>4 (5)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Berenil</td>
<td>&lt;3 (5)</td>
<td>&lt;3 (5)</td>
</tr>
<tr>
<td>Ethidium</td>
<td>4 (5)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>Antrycide</td>
<td>&lt;3 (5)</td>
<td>&lt;3 (5)</td>
</tr>
<tr>
<td>Suramin</td>
<td>&lt;3 (4)</td>
<td>&lt;3 (4)</td>
</tr>
<tr>
<td>Cordycepin</td>
<td>3 (8)</td>
<td>3 (7)</td>
</tr>
</tbody>
</table>

*Parentheses indicate titers for temporary abolition of infectivity.*
have been acquired, since it is unlike that exhibited by newly isolated strains (52). Cross-resistance between arsenicals and acriflavine has long suggested a common link in their trypanocidal action.

The remaining compounds were tested against T. brucei (PU) only, and are grouped under nucleic acid synthesis inhibitors (Table 2), adenosine deaminase inhibitor effects (6), amino acid antagonists, polyanions, and related compounds (Table 3), and miscellaneous inhibitors. Only compounds with significant trypanocidal titers are tabulated.

With all agents found inactive in vitro, cellular access is a largely unknown factor affecting interpretation. The known activity of such compounds on other types of cell has been taken to imply that similar access to the interior of the trypanosome also occurs; this assumption is obviously of limited validity.

**Nucleic acid synthesis inhibitors.** The nucleic acid synthesis inhibitors are grouped according to likely inhibition loci (17) as indicated in Table 2; preliminary tests of some compounds have been reported earlier (51, 56). More compounds with high activity were found in this group of inhibitors than in the other groups tested. As a likely reflection of the parasite’s dependence on exogenous adenine (52), 7 of 11 adenine analogs were actively inhibitory.

The uniquely high trypanocidal titer of daunorubicin places it, with nucleocidin, among the most highly active trypanocidal substances so far described. Unfortunately, it is inactive in vivo, either alone or as a DNA complex (44), in single or multiple maximum tolerated doses. Rapid metabolic degradation (57) is the most likely reason, but doxorubicin, considered to be more stable in this respect, was also inactive in vivo under the same test conditions as daunorubicin.

All those substances in Table 2 which, in other cell systems, are known to inactivate template

<table>
<thead>
<tr>
<th>Inhibition locus</th>
<th>Inhibitor</th>
<th>Trypanocidal titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Motility</td>
</tr>
<tr>
<td>Nucleotide synthesis</td>
<td>Azaserine</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>Arabinosyl adenine</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Adenosine N-oxide</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Virazole</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>Allopurinol</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Nucleotide incorporation into polynucleotides</td>
<td>Cordycepin</td>
<td>4–5*</td>
</tr>
<tr>
<td></td>
<td>Nucleocidin</td>
<td>4–5, 3</td>
</tr>
<tr>
<td></td>
<td>Puromycin aminonucleoside</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>8-Azaguanine</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>2-Fluoroadenosine</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Template DNA inactivation</td>
<td>Acriflavine</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Ethidium</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Miracil D</td>
<td>3</td>
</tr>
<tr>
<td>Intercalative</td>
<td>Actinomycin D</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Daunorubicin</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Nogalamycin</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td>Chromomycin A</td>
<td>&lt;4</td>
</tr>
<tr>
<td>RNA synthesis</td>
<td>Distamycin A</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>Mitomycin C</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8-Hydroxyquinoline</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>HBB</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Ribosome function</td>
<td>Puromycin</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>Emetine</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Chlorotetracycline</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>3</td>
</tr>
</tbody>
</table>

* Parentheses indicate titers for temporary abolition of infectivity.

* Earlier estimates (15).
An appreciable effect was also found in vivo on both the toxicity and activity of cordycepin. Mice survived single intraperitoneal injections of EHNA alone in doses up to 100 mg/kg, and of cordycepin alone in doses up to 180 mg/kg, but the mortality after combined treatment showed increased toxicity of at least fivefold; presumably retention of an intact 6-NH₂ group enhances both mammalian and parasite cytotoxicity.

The degree of enhancement by EHNA of cordycepin trypanocidal activity in vivo is significant, but insufficient to induce fully curative activity (survival > 30 days) with doses of cordycepin (6) that are noncurative alone.

Guanine nucleosides have been reported (19) to potentiate the antitumor activity of several adenosine analogs. In vitro, 0.1 mM guanosine had no enhancing effect on the motility or infectivity (total abolition) titers of cordycepin, but infectivity was temporarily abolished at higher titers than with cordycepin alone. In vivo, an intraperitoneal injection of 200 mg of guanosine per kg did not further enhance the potentiative effects of single or multiple injections of EHNA (3.1 mg/kg) on the activity of cordycepin (12.5 mg/kg).

Cordycepin-N-oxide is a deaminase-resistant derivative which slowly releases cordycepin intracellularly (15). Tests in infected mice showed that toxicity and trypanocidal activity in the single intraperitoneal dose range of 50 to 400 mg/kg were similar to those of cordycepin.

### Amino acid antagonists

Exogenous amino acids markedly prolong the survival of *T. rhodesiense* in serum-free media (55), suggesting trial of a range of specific amino acid antagonists. Only 2 of 13 showed activity, D-C-allyl-glycine (anti-glycine) and D-α-amino butyric acid (anti-valine). Neither had any effect on motility, but the former had a temporary effect on infectivity at a titer of 4, and the latter had a similar effect at appreciably higher titers (6 to 8). Since the effects of both were on infectivity only, some interference with protein anabolism is likely, as suggested for the activity of D-glutamic acid (see below). Little is known of specific amino acid uptake and metabolism in bloodstream trypanosomes, but two separate carrier systems for neutral amino acids have been delineated in *T. brucei* (47), one including glycine and one valine. Although threonine has been implicated as a lipid synthesis source in *T. brucei* culture forms (27), the threonine antagonist, DL-β-hydroxyvaline, was inactive.

Other inactive antagonists (with target amino acids in parentheses) were: taurine (alanine), L-
O-methylthreonine (leucine), L-canavanine (arginine), S-2-aminoethyl-cysteine (lysine), D-ethionine (methionine), DL-m-fluoro-phenylalanine (phenylalanine), DL-1,2,4, triazole-3-alanine (histidine), DL-7-azatryptophan (tryptophan), thio- prolinate (proline), DL-α-methylserine (serine), D-norvaline (valine), D-cycloserine (D-alanine > L-alanine), and cycloleucine (valine). The last two compounds are known to have, respectively, antibacterial (17) and (as WR 14997) antimalarial activity (3).

Polynions and related compounds. The trypanochrome suramin is a polybenzenoid hexa- sulfonate with diverse biological effects characteristic of polyanions in general. Its ability to block macrophage lysosome-phagosome fusion (21) is shared by certain sulfatide lipids (18) and by dextran sulfates (P. D’Arcy Hart and M. A. Young, personal communication). To check a possible relation between lysosomal fusion inhibition and trypanocidal activity, a number of polyanions and related compounds were tested in vitro and, in some cases, in vivo (Table 3). In Table 3, activities are quoted on a weight rather than on a molar basis, since most of the compounds are macromolecules.

Table 3 shows that the polyanions, like suramin, had generally less effect on motility than on infectivity. There was no obvious correlation between trypanocidal activity and ability to inhibit macrophage lysosome-phagosome fusion. Dextran sulfate, poly-D-glutamic acid, and polyethylene sulfonate showed permanent effects on infectivity, and dextran sulfate was especially active in temporary inhibition of infectivity, at a level equal to that of suramin, but the constituent sulfonated marker dye alone, Cibacron blue F3GA, and a related dye, Cibacron brilliant blue BRP, were inactive. D-Glutamic acid was as active as suramin in permanent suppression of infectivity, but was inactive in vivo.

Other polyanions of varying type that had little or no effect were heparin, hyaluronic, polygalacturonic, and algic acids, chondroitin sulfate, carrageenan, and polyuridylic acid. The activity of dextran sulfate appears to depend on sulfation, since the unsubstituted or cation-sub- stituted dextrans were inactive.

Activity in vitro was not confined to anions; the cationic lysine monomers and poly-L-lysine inhibited infectivity permanently, and high activity was not restricted, as in the glutamic acids, to the D-isomers.

Of the several compounds, apart from suramin, which permanently inhibited infectivity in vitro, only poly-D-glutamic acid suppressed the parasitemia of infected mice after intraperito-

neal treatment; the effect was temporary and was achieved only at the maximum tolerated dose (50 mg/kg). Polyethylene sulfonate was not tested.

Miscellaneous inhibitors. The compounds in this group are known to act, in various cells, on specific functions not so far considered, but likely to occur also in the trypanosome.

The following compounds were inactive (with metabolic targets in parentheses): 2-deoxyglucose and 5-thio-D-glucose, in both aerobic and anaerobic cultures (glycolysis); ouabain and betaine sulfate (transport adenosine triphosphatase); nigerin and monensin (ion translocation); cytochalasin B (microfilaments); procaine (“membrane structure”); laevamisole (anthelmintic; primaquine (antimalarial, anti- T. cruzi (36)); and metranidazole (antitoxic).

None of the active compounds is highly trypanocidal, but they give some indication of vulnerable metabolic loci. Pathogenic African trypanosomes have high aerobic glycolysis rates, and the bioflavonoid, quercetin, which is a potent inhibitor of membrane adenosine triphosphate responsible for high aerobic glycolysis in some tumor cells (43), had a marked effect on infectivity only; this was suppressed permanently at a titer of 5 and temporarily to a titer of >8. Since quercetin is strongly bound by serum protein, the effect was demonstrable, as in tumor cells, only by incubation in serum-free medium. Disulfiram (tetraethylthiuram disulfide) has been reported (7) to interfere with threonine metabolism in trypanosomes and to be an active inhibitor of the growth of culture forms of T. brucei (50% inhibition at 0 to 7 μM). On T. brucei (PU), disulfiram shows a titer of 6 against motility and temporary abolition of infectivity up to a titer of 5; effects on T. brucei bloodstream forms appear to have been inconclusive (7). In T. brucei (PU)-infected mice it produced only a slight prolongation of the infection. Colchicine and vinblastine, which affect microtubules, had a temporary effect on infectivity at titers of 4; vinblastine also affected motility at this titer. The longitudinal microtubules which characterize the trypanosome pellicle are likely targets for these compounds; cytochalasin B, attacking microfilaments primarily, is inactive. Amantidine, which affects viral penetration at cell surfaces, had a temporary effect on infectivity only, at a titer of 4; the cell-surface-active antibiotic tyrothricin also showed some activity with a titer of 3 against motility, and temporary abolition of infectivity to a titer of 5. The antitumor dioxopiperazine compound ICRF 159 (6) is one of a series of relatively
nonpolar derivatives of ethylenediaminetetraacetic acid which appear to act early in mitosis. It produced temporary abolition of infectivity to a titer of 4, but had no effect on motility; the homolog ICRF 192, which has no antitumor activity (8), was also without trypanocidal activity. ICRF 159 was inactive in vivo.

**DISCUSSION**

Although this survey does not demonstrate a close correlation of in vitro and in vivo activities and has not shown any compounds with exceptional in vivo activity, the high in vitro activity of several compounds suggests some potentially vulnerable loci of therapeutic attack. Of these loci, RNA synthesis is especially susceptible, and its inhibition is probably the common factor in the antitumor and antiviral activity displayed by many of the most active compounds in Table 2; interference in RNA synthesis by several trypanocides is indicated by the induction of specific nucleolar lesions in drug-treated trypanosomes (54). The cell content of RNA is higher and more continuously synthesized than that of DNA, so that trypanosomal RNA synthesis is likely to be particularly susceptible to inhibition. This assumption is supported by the inactivity of agents known to attack nuclear DNA or DNA synthesis (cyclophosphamide, methyl nitrosoquguanidine, and hydroxyurea) and by the exceptionally high activity (titers of 6 to 10) of (i) the antitumor antibiotics daunorubicin, doxorubicin, chromomycin A3, actinomycin D, and mitomycin C (also a DNA synthesis inhibitor); (ii) the adenine nucleosides tubercidin, cordycepin, and nucleocidin; and (iii) the chelator 8-hydroxyquinoline (14). On intact cells, this last agent probably acts as a permeative toxic iron chelate (2) catalyzing oxidation of essential thiols; the inactivity of the more polar 8-hydroxyquinoline sulfonate suggests that the toxic effect is intracellular. All these compounds are active inhibitors of RNA synthesis in other eucaryotic cells.

Daunorubicin and doxorubicin have been reported active on *T. equiperdum* in vivo (22) and inhibitory for a crithidial RNA polymerase. The structural difference between the two compounds is minimal (57), but daunomycin appears to be the more efficient DNA intercalator, accounting possibly for its higher infectivity titer. Both compounds appear to have a stable storage life of a year; marked loss of trypanocidal activity was noted in samples kept for longer periods. The high incomplete activity of infectivity on the related antibiotic, nogalaminycin, is possibly due to its relative insolubility.

Of the inhibitors of nucleotide utilization and of incorporation into polynucleotides, the majority with trypanocidal activity were adenosine analogs, doubtless reflecting the parasite's dependence on exogenous adenine; arabinosyl adenine, for example, was active, but the cytosine analog was not. Three adenine nucleosides (cordycepin, nucleocidin, and puromycin) have been considered sufficiently active to warrant field trial in Africa (52, 53). 2-Fluoroadenosine and 8-azaguanine had a minor effect on infectivity, but the 8-azapurines, 8-azaadenine and 8-azaxanthine, had no activity; the inactivity of the adenosine analog, formycin A, may be related to its C-nucleoside structure (29), since the C-nucleoside analog of cordycepin also appears to be biologically inactive (9).

In the absence of purine biosynthesis, the inactivity of known biosynthesis inhibitors (6-mercaptopurine and its riboside, hadacidin, psicofuranine, decoyinine, and trimethoprim) is predictable. The activity of azaserine, the glutamine antagonist, is of interest since it is known to inhibit growth of *T. equiperdum* (23), where interference with pyrimidine rather than purine biosynthesis was suggested. Aside from this low activity, pyrimidine biosynthesis appears here to be inaccessible to such known inhibitors as 6-azauridine, iododeoxyuridine, 5-fluorouracil, trimethoprim, and arabinosyl cytosine, which were all inactive; 6-azauracil but not 6-azauridine inhibits pyrimidine biosynthesis and has a growth-suppressive effect in *T. equiperdum* (39). Detectable effects on infectivity were shown by two compounds affecting purine utilization, allopurinol and Virazole (ribavirin) (42). Allopurinol, a close analog of hypoxanthine and primarily a hypoxanthine inhibitor, had no effect on *T. rhodesiense*, suggesting that *T. brucei* (PU) may be more dependent on exogenous purines such as hypoxanthine, known to be a precursor for the salvage synthesis of adenosine monophosphate in *T. gambiense* (41); it was inactive in vivo. The inhibitory effect of allopurinol on the growth of the trypanosomatid *Crithidia fasciculata* (13) at a concentration of 1 μg/ml and on *Leishmania braziliensis* at 5 to 20 μg/ml (35) is comparable to its temporary inhibition of *T. brucei* (PU) infectivity at 1 μM (Table 2). The activity of the antiviral triazole riboside, Virazole, an adenine analog, may be related to its known inhibition of the metabolism of inosine monophosphate, another salvage synthesis precursor (41); a related phytotoxic adenine analog, 3-amino-1,2,4-triazole (15), was shown earlier to be inactive in vitro (51). Theophylline, a nucleoside transport inhibitor, and atractyloside, which inhibits nu-
nucleoside/nucleotide translocation, were both inactive.

The inactivity of rifampin and streptomycin, affecting the RNA polymerase enzyme (not the primer), and of nalidixic acid, affecting DNA synthesis, is explicable in view of their known selective action on procaroytes. α-Amanitin, which attacks the nuclear but not the nucleolar RNA polymerase of eucaryotes, including C. fasciculata (22), was also inactive. If the trypanosomal enzyme is atypical, it may represent a vulnerable locus for selective therapeutic action.

Four compounds known to have specific effects on ribosomes (puromycin, emetine, chlortetracycline, and erythromycin) were active, although the puromycin effect is more likely to be as an adenosine analog on RNA synthesis, rather than on that of ribosomal peptides. Chlortetracycline may act additionally, if not primarily, by chelation of Ca²⁺ ion, in view of the activity of EGTA [ethylenediglycol-bis(β-aminoethyl ether)-N,N-tetraethylic acid] and other Ca²⁺-complexing agents (see below); oxytetracycline has been reported (34) to be much more active on T. congolense. Since erythromycin, like the inactive chloramphenicol, acts selectively on procaroytic ribosomes, its activity here must be due to other factors. Of five test compounds, including three in Table 2, known to act on eucaryotic or on both procaryotic and eucaryotic ribosomes (46), cycloheximide and aurintricarboxylic acid alone were inactive. The advent of new adenosine deaminase inhibitors can extend the trypanocidal as well as the antitumor activity of adenosine nucleosides, although increased toxicity may create problems. The possibilities of interference with amino acid uptake and metabolism remain to be explored and depend largely on more detailed study of the normal mechanisms involved (see ref. 32).

Anionic trypanocidal compounds are relatively rare, and further investigation of them is warranted because they appear generally active against trypanosomes that have become resistant to the common cationic trypanocides. Although the compounds in Table 3 are not highly active, the polyanions may, like suramin, be of use in forming prophylactic salts with cationic drugs.

Each of the active groups has characteristics which may account for the activity observed. The dextran sulfates may affect growth by altering charge distribution and by entrapment of Ca²⁺ ions at the cell surface (30) and possibly internally after endocytosis. The abnormal D-glutamic acids may interfere with protein synthesis, perhaps more effectively as an endocy-}

tosed protease-resistant polymer. Poly-L-lysine has antiviral, antibacterial, antifungal, and antitumor activity (26) and appears to increase cell permeability by surface charge redistribution. Against trypanosomes, the polylolines were much more active on motility, suggesting a surface effect, than the polyglutamic acids or most of the other polyanions tested; the motility titer for poly-L-lysine is comparable to levels quoted for antibacterial activity (26). Both types of polyanion appear to share an ability to interfere with membrane ion transport, by cation displacement or sequestration; this common property may account for the similar infectivity titers of the two ionic groups (Table 3).

Although several of the properties of suramin (prophylaxis due to avid plasma protein binding, protease-complement inhibition, and lysosomotropism) are typical non-specific polyanion characteristics, its trypanocidal action is highly specific, since the demethylated analog is inactive, although still capable of inhibiting lysosome-phagosome fusion in the macrophage system (P. D'Arcy Hart and M. A. Young, personal communication). Lysosomal interaction is thus only one aspect of the cytotoxic action of suramin, but the other trypanocidal polyanions may owe their effect primarily to this function. The dextran sulfates, like suramin, induce formation of multinaucleate trypanosomes, suggesting endocytosis followed by inhibition of RNA or protein synthesis.

The “carrier” effect in lysosomotropism, where a large inert molecule is required for endocytosis of an otherwise active but poorly permeative small molecule, is known to occur with plasma-protein-bound suramin. A similar effect may account for the inactivity of unbound Cibacron F3G3A compared with the high activity of blue dextran, where the dye is bound to a macromolecule; Cibacron F3G3A is also inactive in vivo.

Polyions of the kind examined here may be of use as adjuvants to existing trypanocides, either separately or in combination, to form sparingly soluble complexes for depot prophylaxis (20, 49) or toxicity reduction (58). Some may be suitable for preparation of biodegradable lysosomotropic drug complexes like the DNA complexes of phenanthridinium trypanocides (45) and of dauerobicin (44).

Among the miscellaneous compounds tested, the activity of the glycolysis inhibitor quercetin suggests that related bioflavonoids, which are not inactivated by protein binding in serum, might have potential as trypanocides; as with some tumor cells, high aerobic glycolysis would
be a selective locus of inhibition unshared by the host.

The activity of disulfiram may be related to its inhibitory effects on threonine metabolism, but disulfiram can also act as metal ion chelator on reduction. A number of other compounds with metal-complexing properties have trypanocidal activity, such as tetracyclines, 8-hydroxyquinoline, dithizone and 2,2'-dipyridyl (8), EGTA, ethylenediaminetetraacetic acid, ICRF 159, and aromatic hydroxamates; even cordycepin and nucleocidin are capable of chelation. In these examples, metal binding may not be the sole or primary lethal mechanism, but it is another susceptible locus for therapeutic attack. As an example, α-glycerophosphate oxidase in African trypanosomes is parasite specific and metal-ion associated, being susceptible to suramin and to various metal-complexing agents including salicylohydroxamate (31). This agent, although inactive alone on trypanosomes, becomes trypanocidal when combined with glycerol by effectively blocking both aerobic and anaerobic glycolysis (5, 12). The effect was demonstrable with salicylohydroxamate on T. brucei (PU) in our in vitro tests, but the activity of suramin was not enhanced by glycerol under these conditions.

The most active metal-complexing agent tested was 8-hydroxyquinoline (oxine) (Table 2), which also gave similar activity titers against T. rhodesiense; unlike other chelating agents, its effect was rapid and probably intracellular. Its primary affinity for Fe and Cu ions is shared by 2,2'-dipyridyl, dithizone, ethylenediaminetetraacetic acid, and EGTA, but the first three are almost inactive on either T. brucei (PU) or T. rhodesiense. EGTA gave a titer of 5 for temporary abolition of infectivity in T. brucei (PU); the activity of EGTA, which has more specific binding for Ca\(^{2+}\) ion, may indicate that T. brucei (PU) is more dependent on this metal ion than T. rhodesiense. Like chlortetracycline (Table 2), which complexes Ca\(^{2+}\) ion (33), the activity of the two specific Ca\(^{2+}\)-complexing compounds, arsenazo I and III (4), is directed exclusively on T. brucei (PU), against which both show high titers (7 to 8) for temporary abolition of infectivity. The effect is not likely to be due to the presence of arsenic acid groups in the dye molecule in view of the inactivity against T. rhodesiense. Two other indications of Ca\(^{2+}\) ion importance to T. brucei (PU) are (i) the lower activity (10 mg/ml) of ruthenium red, a calcium-transport-blocking agent, on the motility of T. brucei (PU) compared with T. rhodesiense (1 mg/ml) and (ii) the activity (titer of 4 for temporary abolition of infectivity) of the Ca ionophore Bromo-X-537A (38). Lanthanum nitrate (Ca\(^{2+}\) ion binding site competitor), the Ca-complexing agent murexide, and the Ca precipitants oxalate and picrolonic acid were inactive. The distribution of these activities suggests that the intracellular utilization of externally derived calcium, or a related metal cation, is more essential to T. brucei (PU) than to T. rhodesiense, but more specific confirmation is required.

The varied distribution of likely metabolic or structural targets, suggested by the unsuspected trypanocidal activity of a number of inhibitors, indicates that, apart from test and development of compounds related to those found active, combinations of such active compounds with each other or with established trypanocides should also be tested. When the number of active drugs in use is small and their use is restricted by drug resistance, as in cancer chemotherapy, combination chemotherapy becomes a tactic of necessity, emphasized by the fact that during the last 20 years no new, effective drugs have been brought into field use for human or animal trypanosomiasis.

LITERATURE CITED