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COMPARATIVE PROPERTIES OF SCHISTOSOMAL AND FILARIAL DIHYDROFOLATE REDUCTASES*

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Abstract—Dihydrofolate (DHF) reductase activity was detected in crude extracts of four species of adult filarial worms (*Dirofilaria immitis, Litomosoides carinii, Dipetalonema witei*, and *Onchocerca volvulus*) and the adult blood fluke (*Schistosoma mansoni*). The filariae were much richer sources of the enzyme than were the schistosomes. Lyophilized *D. immitis* and *L. carinii* were as rich a source of the enzyme as comparable numbers of their living counterparts subsequently subjected to acetone powdering. Furthermore, extracts of females of both these species contained more than twice as much DHF reductase activity per milligram of protein as those of the males.

The apparent K_m value of the schistosomal and all four filarial reductases for DHF was approximately 5×10^{-6} M, while that of the parasites' reductases for NADPH was in the range of $1\text{–}3 \times 10^{-5}$ M. Their molecular weights were all around 20,000.

Both schistosomal and filarial DHF reductases showed similar patterns of sensitivity to various drugs and generally were less sensitive than the analogous mammalian enzyme to inhibition by most of the presently available reductase inhibitors. Methotrexate and a related 2,4-diaminoquinazoline (CCNSC 529,861) were the most potent inhibitors among the 14 compounds tested, causing 50 per cent inhibition of enzyme activity in the range of 10^{-8} M to 10^{-10} M. However, while these two compounds were roughly equipotent against the schistosomal and mammalian enzymes, the quinazoline derivative was significantly more potent against the filarial enzymes. In terms of the ID_{50}, the parasites' reductases were more sensitive than the analogous mammalian enzyme to inhibition by the 2,4-diaminopyrimidine trimethoprim. On the same basis, the parasites' reductases were more sensitive to suramin, especially that from *O. volvulus*, the ID_{50} in this case being 2×10^{-6} M compared with 7×10^{-5} for the mammalian reductase.

THE VULNERABILITY of protozoan folate metabolism as a target in the chemotherapy of malaria and acute toxoplasmosis is well established. Little is known about folate metabolism in parasitic helminths. Impaired absorption of folate was found in a high proportion of patients infested with hookworm, and it was suggested that the highest concentration of hookworms occurred at an intestinal level where dietary folate is absorbed.[1] On the other hand, it was reported that sulfonamides, known to inhibit synthesis *de novo* of dihydrofolate, improved the symptoms of patients suffering from acute filariasis.[2] Methotrexate, an analog of folate, was found to be toxic against developing larvae of a nematode parasite of insects, *Neoaplectana glaseri, in vitro*[3] and against developing embryos of the filarial parasite of the cotton rat, *Litomosoides carinii, in vivo*.[4] Very recently, Gutteridge *et al.*[5] reported on the presence and pro-

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perties of dihydrofolate (DHF) reductase from the adult stage strongylid nematode parasite of the rat, *Nippostrongylus brasiliensis*.

We proposed to extract DHF reductase (EC 1.5.1.3) from filarial nematodes as well as from the trematode *Schistosoma mansoni*, in view of the likelihood that tetrahydrofolate (THF) coenzymes, particularly N^5, N^{10}-methylene THF, would be crucial to the maintenance of the high reproductive capacity of these parasitic helminths. Any indication that particular classes of chemical compounds are selectively inhibitory against such DHF reductases would be worth exploring further, since there are presently relatively few effective chemotherapeutic agents against schistosomiases and filariases.

MATERIALS AND METHODS

The following living material was subjected to sonication and acetone powdering preceding the extraction of DHF reductase: adult *S. mansoni* (both sexes combined); male and female adult *Dirofilaria immitis* and *L. carinii* (sexes separated); and rat liver.

S. mansoni were removed from the mesenteric and portal veins of infected mice, produced and maintained in our laboratory. *D. immitis* were either removed by us from the right ventricles and pulmonary arteries of infected dogs supplied by Dr. A. Moreland, University of Florida, Gainesville, Fla., or were obtained alive from Dr. R. Jackson, St. Augustine, Fla. *L. carinii* were removed from the pleural cavity of infected jirds (*Meriones unguiculatus*), provided by Dr. J. Hibbard, University of Georgia, Athens, Ga.

The living material was minced in 3 ml of ice-cold physiological saline solution and then was subjected to sonic disruption at −15° using a Sonifier Cell Disruptor, model W185D (Heat Systems-Ultrasonics, Inc., Plainview, L.I., N.Y.) equipped with a microtip. Sonication with a power output of 40 W for two 30-sec intervals separated by a 30-sec interval generally achieved the desired amount of disruption. The sonicate was quickly subjected to acetone powdering at −15°, and the powder was stored in a desiccator at 3° until used. Under these circumstances, the DHF reductase activity in such preparations remained stable for months.

The following lyophilized material also served as sources of enzyme: male and female *D. immitis* and combined sexes of *L. carinii* (provided by Dr. Hibbard); combined sexes of *Dipetalonema witei* and *Onchocerca volvulus* (provided by Dr. A. Capron, Service de Parasitologie, Faculté de Médecine, Lille, France).

Extraction of DHF reductase from acetone powders or triturated lyophilized material was achieved with ice-cold 0·1 M phosphate buffer, pH 7·0. The powdered equivalent of 600–1200 pairs of *S. mansoni* was suspended in 4 ml of buffer to prepare each batch of enzyme, while 80 mg of filarial material and rat liver powder were similarly processed. DHF reductase activity was found in the supernatant fraction after centrifugation at 10^5 *g* for 30 min using a Beckman model L ultracentrifuge.

A modification of a previously detailed method[6] was used to assay DHF reductase activity. The composition of the standard reaction system was: 0·1 M phosphate buffer, pH 7·0, 100 μmoles; 2-mercaptoethanol, 10 μmoles; NADPH, 0·1 mμole; DHF (prepared by the method of Futterman[7]), 0·1 μmole; and extract (usually a 0·1-ml aliquot), in a final volume of 1·0 ml. The assay was carried out at 37° in matched 1-cm Silica cells using a Perkin–Elmer model 202 recording spectrophotometer

equipped with a multiple sampling assembly in a thermostatically controlled compartment. Activity was associated with a decrease in absorbancy at 340 mμ, which was measured during a 10-min period. One unit of enzyme is defined as that quantity of protein which catalyzes the reduction of 1 mμmole of DHF/min, and the number of enzyme units in an extract was estimated by the method of Blakely and McDougall.[8] Protein was measured according to the method of Lowry *et al.*[9] In drug inhibition studies, sufficient enzyme was used to give a control rate of approximately 0·020 absorbancy unit/min.

Methotrexate (MTX) and diethylcarbamazine citrate (Hetrazan) were obtained from Dr. W. Sweeney, Lederle Laboratories, Pearl River, N.Y. Aspartic acid, *N*-(*p*-([(2,4-diamino-5-chloro-6-quinazolinyl)methyl]amino)benzoyl)-,dihydrate, L-(CCNSC 529,861; NSC) is a Parke-Davis compound which can be obtained from the CCNSC, National Cancer Institute, Bethesda, Md. Dr. G. Hitchings, Wellcome Research Laboratories, Research Triangle Park, N.C., provided 2,4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (pyrimethamine; PYR), 2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine (trimethoprim; TRI), and 2,4-diamino-6-butylpyrido [2,3-*d*]pyrimidine (BW 60-212). Dr. E. Elslager, Parke-Davis Research Laboratories, Ann Arbor, Mich., provided 2,4-diamino-6-(3,4-dichlorobenzylamino)quinazoline (H-Q) and 2,4-diamino-6-[(3,4-dichlorobenzyl)-nitrosamino]quinazoline (NO-Q). Dr. G. Lämmler, Institut für Parasitologie and Parasitäre Krankheitender Tiere, Justus Liebig Universität, Giessen, Germany, provided suramin (Bayer 205; SUR), levamisole (L-tetramisole), tricholorphon (Dipterex), cyclohexane-carboxylic acid-*N*-methyl piperazid citrate (HOE 28637a), tetrahydropyrane-carboxylic acid-*N*-piperazid citrate (HOE 29691a), and 2-(2-(4-hydroxyphenyl)-6-benzimidazolyl)-6-(1-methyl-4-piperazyl)-benzimidazole trihydrochloride (HOE 33258). Triamterene (2,4,7-triamino-6-phenylpteridine) was obtained from the Aldrich Chemical Co., Milwaukee, Wis.

For molecular weight estimations by Sephadex gel filtration, a column of Sephadex G-200, 2·5 × 30 cm, equilibrated with 0·1 M phosphate buffer, pH 7·0 (which also served as the eluant), was calibrated with the protein standards included in the Calibration Kit purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. The void volume (V_0) was first determined by means of Blue Dextran 2000. In the first calibration run, 2 ml of a solution containing 10 mg each of aldolase (mol. wt. 158,000) and chymotrypsinogen A (mol. wt. 25,000) was eluted from the column by ascending chromatography, the reverse flow rate of 14 ml/hr being maintained with a Sigma-motor pump (Model AL-2-E). Aliquots of the effluent (3·5 ml) were collected every 15 min, controlled by means of a tandem recycling timer connected to the fraction collector. In the second calibration run, 2 ml of a solution containing 10 mg each of ovalbumin (mol. wt. 45,000) and ribonuclease A (mol. wt. 13,700) was similarly eluted. Sephadex gelfiltration was carried out in a cold room (3°). The elution volume (V_e) of each protein standard was determined by monitoring the position of exit by its absorption maximum.[10] The K_{av} value for each standard was calculated using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, V_t being the bed volume of the column, and the K_{av} values were plotted on the linear scale of semilog graph paper against the corresponding molecular weights on the log scale. After the Sephadex G-200 column was thus calibrated, 2 ml of the schistosomal, the four filarial, and the rat liver crude extracts (generally possessing sufficient DHF reductase activity so that 0·1 ml would

have a rate change of 0·4 absorbancy unit/min) were individually applied and chromatographed, and the enzyme activity in each fraction was measured spectrophotometrically as described above. The K_{av} values were calculated and located on the previously drawn selectivity curve.

RESULTS

Specific DHF reductase activities of crude extracts. As indicated in Table 1, the four species of adult filariae were richer sources of DHF reductase than adult *S. mansoni*. In terms of enzyme units per milligram of protein, crude extracts of mixed populations (males and females, the latter predominating) of *O. volvulus* had the highest sp. act. (50), followed by *L. carinii* (20), *D. witei* (13), and *D. immitis* (10). By contrast, crude extracts of paired adult *S. mansoni* had a sp. act. of 0·6, a value very close to that found for *N. brasiliensis* (0·5).[5] Also included in Table 1 for purposes of comparison

TABLE 1. DIHYDROFOLATE REDUCTASE ACTIVITY IN CRUDE EXTRACTS OF PARASITIC PROTOZOA AND HELMINTHS

Species	Developmental status	Sex	Sp. act.
P. berghei†	RBC stages		2
T. (T.) rhodesiense‡	Bloodstream forms		10
S. mansoni	Adult	Mixed	0·6
N. brasiliensis§	Adult	Mixed	0·5
D. immitis	Adult	Female	14
D. immitis	Adult	Male	6
L. carinii	Adult	Female	25
L. carinii	Adult	Male	9
D. witei	Adult	Mixed	13
O. volvulus	Adult	Mixed	50
Rat liver			2

* Units per milligram of protein. One unit of DHF reductase reduces 1 mμmole DHF/min.
† Reference 11.
‡ Reference 12.
§ Reference 5.

are values reported for DHF reductase in crude extracts of two protozoan parasites, *Plasmodium berghei*[11] and *Trypanosoma* (*Trypanozoon*) *rhodesiense*,[12] and rat liver. The value of two enzyme units per milligram of protein in crude extracts of plasmodia and rat liver is similar to values reported for bacteria,[13,14] chicken liver[15] and murine Ehrlich ascites carcinoma cells.[16] It was reported earlier[12] that the specific DHF reductase activity in various stercorarian and salivarian trypanosomes was as high or higher than that now reported for the filarial worms, but Walter *et al.*[17] have reported a much lower value for *Trypanosoma* (*Trypanozoon*) *gambiense* and *Trypanosoma* (*Herpetosoma*) *lewisi*, approximately 0·5 in each case. The reason for this discrepancy is not known.

Crude extracts of female *D. immitis* and *L. carinii* contained more than twice as much DHF reductase activity per milligram of protein as those of the males. It is unlikely that the higher enzyme content of females is due to contributions from embryos within them, since we were unable to detect DHF reductase activity in extracts of 10^7 *D. immitis* microfilariae.

It is noteworthy that lyophilized *D. immitis* and *L. carinii* were as rich a source of DHF reductase as comparable numbers of their living counterparts subsequently subjected to acetone powdering.

General properties. The schistosomal and filarial DHF reductases closely resembled the analogous enzyme from rat liver in their strong preference for DHF over folate (F) as substrate and NADPH over NADH as cofactor. For example, when equimolar amounts of F were substituted for DHF, the reaction in all cases proceeded at approximately 10 per cent of the maximal rates. Similarly, when NADH replaced NADPH, in the case of the schistosomal enzyme the reaction proceeded at 30 per cent of the maximal rate, in that of the filarial enzymes, 10–15 per cent, and in that of the rat liver enzyme, 20 per cent.

As indicated in Table 2, the apparent K_m values for DHF and NADPH found for the schistosomal and filarial DHF reductases were closely similar, around 5×10^{-6} M and 2×10^{-5} M respectively. With respect to K_m values for DHF, the helminthic

TABLE 2. APPARENT AFFINITIES (K_m) OF DIHYDROFOLATE AND NADPH FOR PROTOZOAL, SCHISTOSOMAL, AND FILARIAL DIHYDROFOLATE REDUCTASES

Species	$K_m FH_2$ ($M \times 10^6$)	K_m NADPH ($M \times 10^6$)
*P. berghei**	2·6	1·3
T. (T.) rhodesiense†	6	14
S. mansoni	7	28
N. brasiliensis‡	5	8
D. immitis	4	15
L. carinii	5	20
D. witei	5	20
O. volvulus	5	
Rat liver	0·2§	5

* Reference 11.
† Reference 12.
‡ Reference 5.
§ Reference 19.

DHF reductases (including that of *N. brasiliensis*)[5] fell between the high extreme reported for the analogous bacterial enzymes ($2{\cdot}5 \times 10^{-5}$ M)[13] and the low extremes reported for those of chicken liver ($2–5 \times 10^{-7}$ M)[15,18] and rat liver (2×10^{-7} M).[19] With respect to K_m values for NADPH, those of the schistosomal and filarial DHF reductases, while very similar to those of trypanosomes[12,17] and bacteria,[13,14] were higher than the values reported for *N. brasiliensis* (8×10^{-6} M),[5] plasmodia ($1{\cdot}3–5 \times 10^{-6}$ M),[11,17] and avian and mammalian liver ($1{\cdot}7–5 \times 10^{-6}$ M).[15,20]

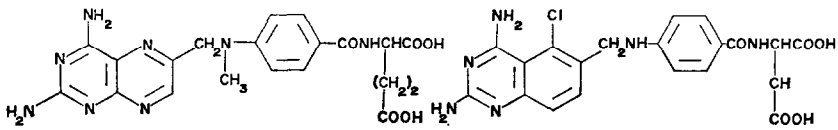
As estimated by their behavior on a calibrated column of Sephadex G-200, the molecular weights of the schistosomal and all four filarial DHF reductases were around 20,000. In this respect, they were closely similar to the analogous enzymes from *N. brasiliensis*,[5] as well as from bacterial,[14,21–23] avian,[15,24] and mammalian[16,24–27] sources. It is noteworthy that the analogous enzymes from protozoa (crithidia,[24] trypanosomes,[24] and plasmodia[11,28]) were found to have markedly higher molecular weights, in the range of 100,000–200,000.

Sensitivity to antifolate compounds and anthelmintic agents. Although all DHF reductases are characterized by extreme susceptibility to inhibition by 4-amino analogs of folate, Burchall and Hitchings[13] were the first to point out that DHF reductases of bacterial and mammalian origin could be distinguished by their differential sensitivity to certain 2,4-diaminopyrimidines and related low molecular weight heterocycles. Characteristic drug inhibitor profiles have since been described for the analogous enzymes from crithidia,[29] stercorarian and salivarian trypanosomes,[12] *P. berghei*,[11] *N. brasiliensis*[5] and mammalian cells.[13] It remained to delineate the drug inhibitor profiles of the schistosomal and filarial DHF reductases. The types of chemical compounds selected for assessment were first of all those with established antifolate activity: the 4-amino-N^{10}-methyl analog of folic acid, methotrexate, and a related 2,4-diaminoquinazoline (CCNSC 529,861);[30] three 2,4-diaminopyrimidines;[13] pyrimethamine, trimethoprim and BW 60-212; two unconjugated 2,4-diaminoquinozolines;[31,32] and the 2,4,7-triamino-6-phenylpteridine, triamterene.[16] Also tested were seven compounds which were either primarily microfilaricidal (diethylcarbamazine, levamisole, trichlorphon, HOE 28637a, HOE 29691a, HOE 33258) or adulticidal (suramin) against *L. carinii* in the multimammate rat, *Mastomys natalensis*.* Suramin, it should be noted, is one of the drugs of choice in the treatment of onchocerciasis.[33]

As could be expected, these helminthic DHF reductases were extremely sensitive to inhibition by the two antifolates resembling folic acid (Table 3). It is of interest that although both methotrexate and CCNSC 529,861 were roughly equipotent on a molar basis against the schistosomal (as well as the mammalian) enzyme, the latter compound was significantly more potent against all four filarial enzymes, especially that of *O. volvulus*. It should, however, be emphasized that with regard to such 4-amino analogs of folate, which may act as stoichiometric inhibitors, comparisons of their potency against DHF reductases from different sources must be interpreted with caution unless the turnover numbers of the enzymes are known, which is not the case in this study.

Both the schistosomal and the filarial enzymes were relatively insensitive to inhibition by the three 2,4-diaminopyrimidines, in contrast to the marked sensitivity of the analogous enzymes from *P. berghei* and *T.* (*T.*) *rhodesiense* to this class of antifolates (Table 4). The schistosomal and filarial enzymes were less sensitive than the analogous mammalian enzyme to inhibition by pyrimethamine and BW 60-212. In terms of the ID_{50}, they were somewhat more sensitive to trimethoprim than the mammalian enzyme, but such small differences disappear if one calculates the apparent K_i values in this instance. It is noteworthy that quite marked differences were found in the sensitivity of the analogous DHF reductases from the four filarial species and that of the closely related nematode *N. brasiliensis*[5] to pyrimethamine and BW 60-212, although their sensitivity to trimethoprim was mutually closely similar (Table 4).

TABLE 3. COMPARATIVE SENSITIVITY OF DIHYDROFOLATE REDUCTASES FROM PARASITIC PROTOZOA AND HELMINTHS TO ANTIFOLATES RESEMBLING FOLIC ACID



METHOTREXATE (MTX)

CCNSC 529,861 (NSC)

Source of enzyme	Concn. ($\times 10^{-8}$ M) for 50% inhibition	
	MTX	NSC
*P. berghei**	0·07	
T. (T.) rhodesiense†	0·1	0·04
S. mansoni	0·07	0·01
N. brasiliensis‡	0·15	
D. immitis	0·2	0·03
L. carinii	1	0·1
D. witei	3	0·4
O. volvulus	3	0·04
Rat liver	0·2	0·3
Human leukemia cells§	0·08	0·06

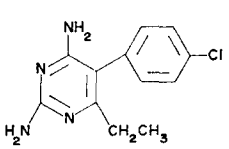
* Reference 11.
† Reference 12.
‡ Reference 5.
§ Reference 30.

As indicated in Table 5, the *N*-nitroso derivative of a 6-dichlorobenzyl-substituted 2,4-diaminoquinazoline was generally significantly more potent than the parent compound against the DHF reductases of the parasites. The exception was the DHF reductase of *O. volvulus* which did not exhibit differential sensitivity to the two homologs, a property it shared with the analogous mammalian enzyme.

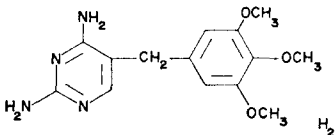
The 2,4,7-triamino-6-phenylpteridine, triamterene, proved to be an extremely weak inhibitor of the helminthic DHF reductases, causing 50 per cent inhibition of maximal activity in the concentration range of 4–7 $\times 10^{-5}$ M. On the other hand, the ID_{50} of triamterene against the rat liver enzyme was $1{\cdot}5 \times 10^{-6}$ M.

None of the primarily microfilaricidal drugs (diethylcarbamazine, levamisole, trichlorphon, HOE 28637a, HOE 29691a or HOE 33258) significantly inhibited any of the DHF reductases studied at concentrations as high as 10^{-4} M. By contrast, suramin showed significant inhibitory activity, and in terms of the ID_{50}, the DHF reductases of the parasites were more sensitive to this drug than was the mammalian enzyme. As indicated in Table 6, suramin was for the most part a relatively weak inhibitor of DHF reductase, causing 50 per cent inhibition of maximal activity in the range of 10^{-5} M or higher. The outstanding exception was the DHF reductase of *O. volvulus* which was inhibited by 50 per cent in the presence of 2×10^{-6} M suramin, being thus 35 times more sensitive to this drug than the analogous mammalian enzyme in terms of the ID_{50}.

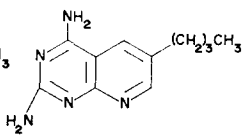
TABLE 4. COMPARATIVE SENSITIVITY OF DIHYDROFOLATE REDUCTASES FROM PARASITIC PROTOZOA AND HELMINTHS TO 2,4-DIAMINOPYRIMIDINES



PYRIMETHAMINE (PYR)



TRIMETHOPRIM (TRI)

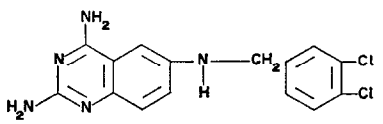


BW60-212

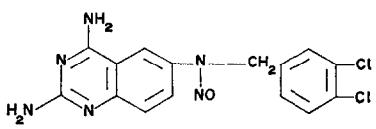
Source of enzyme	Concn. ($\times 10^{-8}$ M) for 50% inhibition		
	PYR	TRI	BW 60–212
*P. berghei**	0·05	7	1·7
T. (T.) rhodesiense†	20	25	60
S. mansoni	700	1000	1000
N. brasiliensis‡	70	1000	50
D. immitis	1500	2000	1000
L. carinii	1000	1000	300
D. witei	4000	5500	800
O. volvulus	4000	4000	200
Rat liver	200	40,000	50

* Reference 11.
† Reference 12.
‡ Reference 5.

TABLE 5. COMPARATIVE SENSITIVITY OF DIHYDROFOLATE REDUCTASES FROM *T. (T.) rhodesiense* AND PARASITIC HELMINTHS TO 6-SUBSTITUTED 2,4-DIAMINOQUINAZOLINES



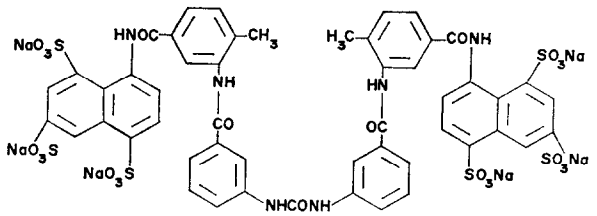
(H-Q)



P-D CI679 (NO-Q)

Source of enzyme	Concn. ($\times 10^{-8}$ M) for 50% inhibition	
	H-Q	NO-Q
T. (T.) rhodesiense	200	20
S. mansoni	500	40
D. immitis	200	20
L. carinii	200	10
D. witei	700	100
O. volvulus	100	100
Rat liver	60	60

TABLE 6. COMPARATIVE SENSITIVITY OF DIHYDROFOLATE REDUCTASES FROM *T.* (*T.*) *rhodesiense* AND PARASITIC HELMINTHS TO SURAMIN



Source of enzyme	Concn. ($\times 10^{-8}$ M) for 50% inhibition
	Suramin
T. (*T.*) *rhodesiense*	3000
S. mansoni	3000
D. immitis	1000
L. carinii	1000
D. witei	1500
O. volvulus	200
Rat liver	7000

In view of the admonition of McCullough and Bertino[34] that purified DHF reductases should be used in measuring inhibition produced by analogs, we measured the sensitivity of the schistosomal and filarial enzymes, following their partial purification by passage through Sephadex G-200, to every compound in our study. The ID_{50} value in every case was identical to that previously determined using the original crude preparation.

DISCUSSION

The detection of DHF reductase activity in extracts of *S. mansoni* and filarial worms belonging to four genera raises questions concerning the physiological role of this enzyme in these parasitic helminths and the prospects for using antifolate drugs in the chemotherapy of schistosomiases and filariases.

Various THF coenzymes function in reactions involving interconversions among several amino acids, initiation of peptide chain synthesis, and synthesis *de novo* of purine nucleotides and thymidylate (dTMP). Although further investigation is required to establish the relative importance of these biosynthetic pathways and their "salvage pathway" counterparts in the economy of schistosomes and filariae, there is growing evidence that, in the area of nucleic acid metabolism, the synthesis *de novo* of dTMP is probably the most crucial for their normal growth and the maintenance of their high reproductive capacity.

Recent investigations of *S. mansoni*, *in vitro*[35] and *in vivo*,[36] have indicated that this trematode depends primarily, if not exclusively, upon preformed purines (especially adenine or adenosine) for the synthesis of purine nucleotides. Similar studies have not yet been undertaken with adult filariae, but *D. immitis* microfilariae were found[37]

to incorporate radioactivity derived from ^{14}C-ring-labeled adenine and adenosine but not that from ^{14}C-labeled glycine or formate into purine nucleotides.

On the other hand, at least adult *S. mansoni* and *D. immitis* were able to incorporate radioactivity derived from ^{14}C-ring-labeled uracil and uridine into the DNA fraction as well as that derived from ^{14}C-ring-labeled thymidine (our unpublished results). It is doubtful that the extremely limited amounts of thymidine in mammalian host extracellular fluids[38] would be sufficient *per se*, when converted to dTMP and thence to thymidine-5′-triphosphate (dTTP) by way of the salvage pathway, to meet the needs of these parasites for this vital DNA precursor and allosteric activator of purine ribonucleoside diphosphate reductase.[39]

The major *de novo* biosynthetic pathway leading to dTMP involves the methylation of deoxyuridylate (dUMP), this reaction being catalyzed by thymidylate synthetase with N^5,N^{10}-methylene THF serving as donor of the single carbon substituent as well as the required hydrogen atoms.[40] As a consequence of the reaction, DHF is regenerated and becomes available for reconversion to THF in the presence of DHF reductase. Thus, inhibition of DHF reductase would predictably interfere with the thymidylate synthesizing system.

The potential efficacy of antifolate compounds as chemotherapeutic agents depends upon the interaction of various factors, including the amount and location of DHF reductase and its normal substrate, DHF, within target cells and those of the host, the comparative affinities of target cell and host enzymes for substrate and cofactor, the ability of inhibitors to reach the target enzyme, and the differential sensitivity of target cell and host enzymes to inhibition by such agents.

We found that crude extracts of *S. mansoni* possessed much less DHF reductase activity than those of four species of filarial worms. While the amounts of DHF in these parasites were not measured, in other organisms it was found that tissues with the largest amount of DHF reductase were also those having the largest stores of folate derivatives.[41,42] Although, generally, cells resistant to the action of antifolate compounds contain higher than normal amounts of DHF reductase,[43–46] Roberts *et al.*[47] could find no correlation between sensitivity to methotrexate and enzyme concentration *per se* in a variety of transplantable rodent tumors which had never been exposed to antifolates previously.

Of more probable predictive value than enzyme concentration *per se* is the drug sensitivity profile of DHF reductase from target cells of the parasite compared with that of the analogous enzyme from host tissues. If the enzyme of a parasite exhibits significantly greater sensitivity to a particular class of inhibitor and provided enough of the inhibitor can reach the enzyme within the intact organism, such an agent is more likely to exert a chemotherapeutic action. An excellent example supporting this view is provided by Ferone *et al.*[11] who presented convincing evidence that the antimalarial activity of pyrimethamine could be accounted for by the over 1000-fold greater binding of this drug to the parasite than to the host enzyme. However, such a large differential sensitivity to an enzyme inhibitor need not be obligatory for effective chemotherapeutic action. The anti-schistosomal activity of organic antimonials has been ascribed to the 80-fold greater sensitivity of schistosomal phosphofructokinase to inhibition by these agents than the analogous mammalian enzyme.[48] In this case, the consequences of this selectively toxic action at the enzyme level are further amplified at the physiological level because of the much greater

dependence of the schistosomes than the host upon phosphorylative glycolysis for energy generation.[49]

We have found that, generally, the schistosomal and filarial DHF reductases were either less sensitive than the analogous mammalian enzyme to available antifolate compounds or, in a few cases, slightly more sensitive. On this basis, one would tend to agree with the opinion of Gutteridge *et al.*[5] that "the prospects of using dihydrofolate reductase inhibitors in the chemotherapy of helminth infections are not encouraging". However, several observations in this area deserve special mention.

The significantly greater sensitivity of the filarial reductases (especially that of *O. volvulus*) to the conjugated 2,4-diaminoquinazoline compound CCNSC 529, 861 than to methotrexate is of interest, in view of the finding that the latter inhibited embryonal development *in utero* of *L. carinii* in cotton rats.[4]

The schistosomal and filarial reductases were, in terms of the ID_{50}, up to 40 times more sensitive to the 2,4-diaminopyrimidine trimethoprim than the analogous mammalian enzyme. Unfortunately, trimethoprim proved ineffective against *D. immitis* in the dog and *L. carinii* in the jird* as well as against *N. brasiliensis* in the rat.[5] If lack of potency *in vivo* were found to be only a matter of too small a difference in sensitivity between parasite and host enzyme, then further investigation of trimethoprim homologs might be indicated in the hope that related compounds with greater selective toxicity might be discovered. However, it should be noted that salivarian trypanosomal DHF reductases are almost 1000 times more sensitive to trimethoprim than the analogous mammalian enzyme and yet this drug exerted no anti-trypanosomal activity *in vivo*.[12]

In view of the finding that the *N*-nitroso derivative of 2,4-diamino-6-(3,4-dichlorobenzylamino)quinazoline was active against *L. carinii* in the jird,* it was of considerable interest to discover that the nitroso substituent increased by 10-fold the inhibitory potency of the parent compound against the DHF reductases of *S. mansoni*, *D. immitis* and *L. carinii* (Table 5). There was less enhancement of potency due to the nitroso substitution against the *D. witei* reductase and no enhancement of potency against the *O. volvulus* reductase as well as the mammalian reductase. This finding suggests that it might be worthwhile to investigate other members of this family of diaminoquinazolines for correlations between selective DHF reductase inhibitory potency and anthelmintic activity.

Suramin was found to be curative against *L. carinii* infections in the multimammate rat, *Mastomys natalensis*,† and it is established as one of the drugs of choice against *O. volvulus* infections in man,[33] and also early *T.* (*T.*) *gambiense* and *T.* (*T.*) *rhodesiense* infections in man.[50] The precise modes of its anti-parasitic actions remain uncertain. Although this drug was found to inhibit a number of enzymes *in vitro* including various oxidases and kinases, suramin is not a general enzyme poison and most of the non-proteolytic enzymes tested were unaffected by the drug.[51] Hexokinase was the most sensitive of the enzymes tested, being inhibited by 50 per cent at a concentration of suramin in the range of 2×10^{-5} M.

Our findings indicate that DHF reductases of schistosomal and filarial (as well as trypanosomal) origin are among the most sensitive enzymes, of all those tested, to

* Dr. J. Hibbard, personal communication.
† Dr. G. Lämmler, personal communication.

inhibition by suramin (Table 6). Especially noteworthy is the sensitivity of the *O. volvulus* reductase, which was inhibited by 50 per cent in the presence of 2×10^{-6} M suramin. Even after taking into account the extensive plasma protein binding of suramin,[52] this concentration of unbound drug is likely to be maintained following the usual dose regimen (1 g intravenously once weekly for 5 weeks) administered to patients suffering from onchocerciasis. Thus, it is conceivable that the clinical efficacy of suramin against this important filarial infection may be due in part to its ability to inhibit onchocercal DHF reductase.

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