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EFFECTS OF HYCANTHONE ON RAPIDLY PROLIFERATING CELLS

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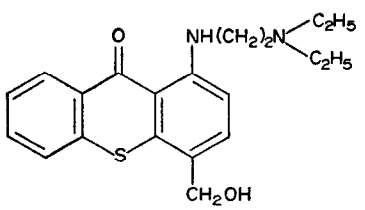
Abstract—The effects of hycanthone on various types of rapidly proliferating tissue, including tumor cells *in vitro* and *in vivo*, phytohemagglutinin (PHA)-stimulated lymphocytes, and embryonic cells, have been investigated. Hycanthone was cytotoxic to several tumor cell lines in tissue culture and exerted antitumor activity *in vivo* against Walker 256 carcinosarcoma, leukemia L1210 and mast cell P815. Human PHA-stimulated lymphocytes incubated in the presence of hycanthone showed a 50 per cent depression in mitotic index, and a marked increase in number of chromosomal aberrations. Hycanthone administered to pregnant mice was not teratogenic, but its embryotoxicity was manifested by an increased incidence of intrauterine death and a decrease in average fetal body weights. Mechanism studies have shown that hycanthone inhibits both DNA and RNA, but not protein synthesis, in human PHA-stimulated lymphocytes. Furthermore, hycanthone was found to inhibit aldehyde oxidase and this inhibition was greater than that previously reported for the *N*-alkylphenothiazines. These studies suggest that hycanthone may have promise as an antitumor agent, either alone or in combination with antitumor agents known to be inactivated by aldehyde oxidase.

HYCANTHONE is a compound with potent antischistosomal activity against *Schistosoma mansoni* and *Schistosoma haematobium* in both animals and man.[1,2] Clinical trials in South America and Africa have shown that the compound is efficacious and relatively nontoxic.[2,3] In structure, hycanthone is a hydroxymethyl derivative (Fig. 1) of



Hycanthone: 1-{[2-(Diethylamino)ethyl] amino}
4-(hydroxymethyl)-thioxanthen-9-one

FIG. 1. Structural formula for hycanthone.

lucanthone (Miracil D), an antischistosomal agent which has been in use for several decades.[4] However, lucanthone produces undesirable side effects and its usefulness has been limited for this reason.[5] Recently *Aspergillus scleriotum* has been shown to

degrade lucanthone to hycanthone, and hycanthone has been identified in the urine of several animal species following treatment with lucanthone.[6] There is evidence that one of the metabolites of lucanthone, rather than lucanthone itself, is primarily responsible for schistosomicidal activity,[6] and that this active metabolite is indeed hycanthone.[6,7] Many of the toxic effects of lucanthone have been ascribed to the unmetabolized drug.[3]

In addition to its antischistosomal activity, lucanthone has been reported to have antitumor activity in a number of experimental tumors in mice, including Sarcoma 180, leukemia L1210 and adenocarcinomas 755 and EO771.[8] Again, its metabolic conversion to a more active metabolite appears necessary for carcinostatic activity.[9] It is possible that the antitumor activity of lucanthone, like its antischistosomal activity, may also be due to its metabolite hycanthone; Hirschberg *et al.*[10] have shown, however, that hycanthone is equivalent but not superior to lucanthone as an inhibitor of growth and RNA synthesis in L1210 cells. The identity of this carcinostatic metabolite of lucanthone must therefore be regarded as still unsettled.

The present investigation represents an attempt to assess the effects of hycanthone on a variety of types of rapidly proliferating tissue, including tumor cells *in vivo* and *in vitro*, phytohemagglutinin (PHA)-stimulated lymphocytes, and embryonic cells.

EXPERIMENTAL

Drug. Hycanthone was received as the methanesulfonate salt and was the gift of Sterling Winthrop Research Institute, Rensselaer, N.Y.

Cytotoxicity studies. Hycanthone was tested *in vitro* for cytotoxic effects against leukemia L1210, Walker carcinosarcoma 256 and Novikoff hepatoma cells. Leukemia L1210 cells were maintained in static culture in RPMI No. 1630 medium with 20% fetal calf serum.[11,12] Walker 256 carcinosarcoma and Novikoff hepatoma cells were maintained in static culture in RPMI No. 1630 medium with 10% fetal calf serum. Stock cultures of each cell line were counted and diluted so that there were 10^5 cells/ml with a total of 5 ml in each bottle.

Hycanthone was dissolved in physiological saline and volumes of 0·1 ml were added to the 5 ml of cells, resulting in final hycanthone concentrations of 0·01 to 100 μg/ml. Solutions of hycanthone were passed through a 0·45 μ Millipore filter before addition to the culture. The cells were counted by a Coulter counter every 24 hr for 2 days (leukemia L1210 and Walker 256 carcinosarcoma) or 3 days (Novikoff hepatoma). The effect of hycanthone on the growth of each cell line was determined at least twice with duplicates at each concentration, and the results are presented as per cent inhibition as compared to saline-treated controls.

Evaluation of antitumor activity. Leukemia L1210, mast cell P815, leukemia P388, leukemia L5178Y and Walker carcinosarcoma 256 were transplanted intraperitoneally after dilution of the ascitic fluid from donor mice or rats with Locke's solution. Male CDF_1 mice were inoculated with 0·1 ml of a 1 : 1000 dilution of leukemia L1210 ascites, with a 1 : 100 dilution of mast cell P815 ascites, a 1 : 20 dilution of leukemia P388 ascites or a 1 : 1000 dilution of leukemia L5178Y ascites. Sprague–Dawley rats were inoculated with a 1 : 100 dilution of Walker carcinosarcoma ascites. Hycanthone, dissolved in physiological saline, was given daily intraperitoneally beginning 24 hr after inoculation of tumor. Experiments were carried out employing doses of hycanthone ranging from 6 to 150 mg/kg.

Effects on chromosome morphology and mitotic indices of human lymphocytes. The effect of hycanthone on the mitotic index and chromosome morphology of lymphocytes was examined by methods previously described.[13,14] Human lymphocytes were isolated from the peripheral blood of healthy donors. One ml of cell-rich plasma (6×10^6 cells/ml) was mixed with 1 ml of autologous serum (30%) and 4 ml of McCoy's medium, resulting in a final cell concentration of 1×10^6 cells/ml of medium. After the addition of 0·6 mg phytohemagglutinin (PHA, Difco), samples were incubated at 37°. Hycanthone, at concentrations ranging from 5 to 50 μg/ml, was added to the incubation mixtures either at the start of the incubation or 4 hr before harvesting at 48 hr. Chromosome aberrations classified as minor included breaks or fragments; those classified as major were ring chromosomes, dicentrics, fragmentation, chromatid exchanges and pulverization.

Embryotoxicity studies. Hycanthone, at dose levels of 12·5, 25 and 50 mg/kg was administered daily s.c. to pregnant NIH mice on days 6–11 of gestation. Untreated mice and mice receiving saline in equivalent volumes on days 6–11 served as controls. Fetuses were obtained by laparotomy at day 18 of gestation. The number and position in the uterine horns of both live and resorbed fetuses were recorded and the live fetuses were weighed and fixed in 10% formalin. Fetuses were sectioned by the Wilson technique[15] in order to detect viceral abnormalities; one-third of each litter, selected at random, was stained with alizarin red S for detection of skeletal malformations.[16] The average fetal body weights from litters of hycanthone-treated mice were compared to those of litters from untreated or saline-treated controls by Student's *t*-test.[17] Analysis of differences between control and drug-treated litters in regard to intrauterine death, incidence of abnormalities, and sex ratios was made by the chi-square test.[17]

Studies on DNA, RNA and protein synthesis in human lymphocytes. Human lymphocytes were isolated from the peripheral blood of healthy donors and incubated as described above. Studies on DNA, RNA and protein synthesis were performed by measuring uptake of radioactivity after addition of labeled precursors to the incubation mixture. ^{3}H-methyl-thymidine (15·9 Ci/m-mole), ^{14}C-2-uridine (59·8 mCi/m-mole) and ^{14}C-1-L-leucine (262 mCi/m-mole) were obtained from New England Nuclear Corp., Boston, Mass. Hycanthone, at concentrations ranging from 0·05 to 50 μg/ml, was added to cells at the beginning of the incubation. The labeled precursors (0·5 μCi/ml of incubation mixture) were added to the incubation mixtures 2 hr before harvesting at 48 hr. The cells were washed and macromolecules precipitated as previously described.[18] Radioactivity in DNA, RNA and protein was measured by liquid scintillation spectrometry and all results were corrected for quenching. In addition, studies were carried out in which hycanthone (20 μg/ml, final concentration) and labeled precursors were added to human lymphocytes at various time intervals after addition of PHA. The cells were harvested 2 hr after the addition of hycanthone and labeled precursors; DNA, RNA and protein synthesis were measured as above.

Inhibition of aldehyde oxidase. Rabbit liver aldehyde oxidase was purified by heat treatment, ammonium sulfate fractionation, and adsorption on and elution from calcium phosphate gel, as described by Rajagopalan *et al.*[19] Protein determinations were made according to the method of Lowry *et al.*[20] The specific activity of the aldehyde oxidase preparation was measured by following spectrophotometrically at 340 nm the conversion of methotrexate to 7-hydroxymethotrexate at 37°.[21] This

activity was calculated to be 90 nmoles methotrexate oxidized/mg of protein/min. This represented a purification factor of approx. 84 over the activity of the supernatant fraction from the crude liver homogenate.

RESULTS

Cytotoxic effects of hycanthone. Table 1 shows the effect of hycanthone on the growth of L1210, Walker carcinosarcoma 256 and Novikoff hepatoma cells *in vitro*. Novikoff hepatoma cells appear to be most sensitive to the inhibitory effects of hycanthone, since hycanthone at a concentration of 10 μg/ml inhibited their growth

TABLE 1. CYTOTOXIC EFFECTS OF HYCANTHONE AGAINST CELLS IN TISSUE CULTURE*

Concn (μg/ml)	% Inhibition of control growth		
	Leukemia L1210	W256 Carcinosarcoma	Novikoff hepatoma
100	89·0	80·0	97·0
50	85·9	82·5	96·0
10	58·4	70·3	90·1
1	12·3	10·5	70·0
0·1	5·0	5·0	55·2

* Stock cultures of leukemia L1210, Walker 256 carcinosarcoma and Novikoff hepatoma cells were diluted to a concentration of 10^5 cells/ml. Hycanthone was dissolved in physiological saline and volumes of 0·1 ml were added to 5 ml of cells, resulting in final hycanthone concentrations of 0·1 to 100 μg/ml. Cells were counted by Coulter counter 24 hr (leukemia L1210) or 48 hr (Walker 256 carcinosarcoma and Novikoff hepatoma) after addition of hycanthone. The results represent the mean of at least two experiments with duplicates at each concentration and are expressed as per cent inhibition of cell growth as compared to saline-treated controls.

90 per cent compared to controls. At concentrations of 1 μg/ml, their growth was inhibited 70 per cent, and doses of 0·1 μg/ml inhibited this cell line more than 50 per cent. At concentrations of 10 μg/ml, the growth of leukemia L1210 and Walker 256 carcinosarcoma was inhibited approx. 60 and 70 per cent respectively. Doses of 0·1 and 1 μg/ml did not inhibit the growth of leukemia L1210 or Walker 256 cells.

Antitumor activity of hycanthone. The activity of hycanthone against several tumor systems is shown in Table 2. Hycanthone was most effective in prolonging the life-span of rats inoculated with the Walker carcinosarcoma 256, since animals receiving daily i.p. injections of 30–50 mg/kg are alive and tumor free 4 months after inoculation. Hycanthone administered in daily doses of 60 mg/kg increased the median life-span of mice bearing the leukemia L1210 tumor 88 per cent. Hycanthone was also effective in increasing the survival time of mice bearing leukemia P388 and mast cell tumor P815, but was less effective against leukemia L5178Y, increasing the median survival time only 41 per cent in animals inoculated with this tumor.

Effect of hycanthone on chromosome morphology and mitotic indices. Table 3 shows the effect of various concentrations of hycanthone on the mitotic index and chromosome morphology of PHA-stimulated human lymphocytes. When hycanthone was

TABLE 2. ANTITUMOR ACTIVITY OF HYCANTHONE*

Tumor	Range of doses (mg/kg)	Optimum daily dose (mg/kg)	% Increase in median life span
Leukemia L1210	6–150	60–70	88
Leukemia P388	6–100	70	122
Leukemia L5178Y	50–100	60	41
Mast cell P815	25–100	60	70
Carcinosarcoma W-256	30–50	30–50	> 1000†

* Male CDF_1 mice were inoculated i.p. with tumor cells diluted from the ascitic fluid from donor mice carrying leukemia L1210, leukemia P388, leukemia L5178Y or mast cell P815. Male Sprague–Dawley rats were inoculated i.p. with Walker 256 carcinosarcoma ascites cells. Daily i.p. injections of hycanthone began 24 hr after inoculation and treatment was continued until death.

† All rats alive and tumor free 4 months post-inoculation.

added to cells at the beginning of the 48-hr incubation period, the compound decreased the mitotic index to 50 per cent of control value at the lowest concentration (5 μg/ml) tested, and the mitotic index was reduced to zero at concentrations of 20 μg/ml or higher. Less prominent effects on mitotic index were observed when hycanthone was added to the lymphocyte culture 4 hr before the end of a 48-hr incubation. Thus, no depression of mitotic index was observed at hycanthone concentrations less than 25 μg/ml.

Table 3 also shows that when human PHA-stimulated lymphocytes are exposed to hycanthone at concentrations of 5–15 μg/ml for the duration of the 48-hr incubation, there is no increase in incidence of chromosomal aberrations over controls. However, when hycanthone was added to the lymphocyte culture 4 hr prior to harvesting 48 hr, a marked increase in chromosomal aberrations was seen at doses of 20 μg/ml or

TABLE 3. EFFECT OF VARIOUS CONCENTRATIONS OF HYCANTHONE ON PHA-STIMULATED HUMAN LYMPHOCYTES—MITOTIC INDICES AND CHROMOSOMAL ABERRATIONS

Hycanthone concn (μg/ml)	Drug added at zero time				Drug added at 44 hr			
		Aberrations (%)				Aberrations (%)		
	M.I.*	Total	Minor	Major	M.I.*	Total	Minor	Major
Control	18	12	10	2	18	12	10	2
5	10	12	12	0	18	10	10	0
10	5	8	8	0	18	18	14	4
15	0·5	10	10	0	15	14	12	2
20	0				14	28	18	10
25	0				8	30	16	14
50	0				3†			

* Mitotic indices.

† No detailed analysis could be made in these preparations; cells contained very elongated chromosomes, stickiness with chromatid erosion, exchanges and/or breaks.

TABLE 4. EMBRYOTOXICITY OF HYCANTHONE IN PREGNANT MICE*

Treatment	Dose (mg/kg)	No. of mice	Total No. of implantations	Resorptions (No.)	Resorptions (%)	Fetuses Normal	Fetuses Abnormal	Average fetal body wt. (g)
None		11	117	9	7·7	107	1	1·085
Saline		7	78	6	7·7	72	0	1·021
Hycanthone	12·5	7	62	2	3·2	60	0	0·916†
Hycanthone	25·0	5	46	9	19·5	36	1	0·790†
Hycanthone	50·0	10	81	74	91·4	6	1	0·771†

* Hycanthone dissolved in saline or saline alone (0·01 ml/g) was given subcutaneously to pregnant NIH albino mice on days 6–11 of gestation. Fetuses were obtained by laparotomy at 18 days of gestation. The number of live and resorbed fetuses was recorded and live fetuses were weighed and examined for malformations.

† $P < 0.001$.

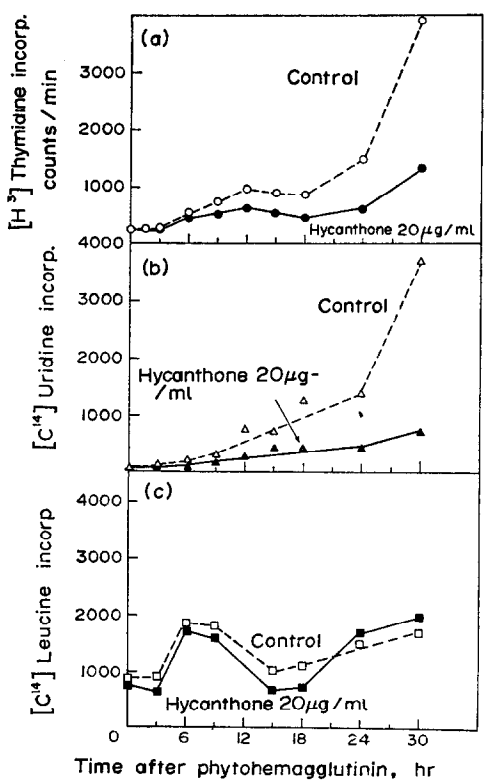


FIG. 3. Effect of hycanthone on DNA, RNA and protein synthesis in human lymphocytes at various intervals after PHA stimulation. Hycanthone (20 μg/ml of incubation medium) and ^{3}H-methyl-thymidine, ^{14}C-1-L-leucine or ^{14}C-2-uridine (0·5 μCi/ml) were added to cultures of human lymphocytes at various intervals following stimulation with PHA. Two hr later, the cells were harvested and radioactive incorporation was measured. Control cultures (open symbols) were incubated with radioactive precusors in the absence of hycanthone.

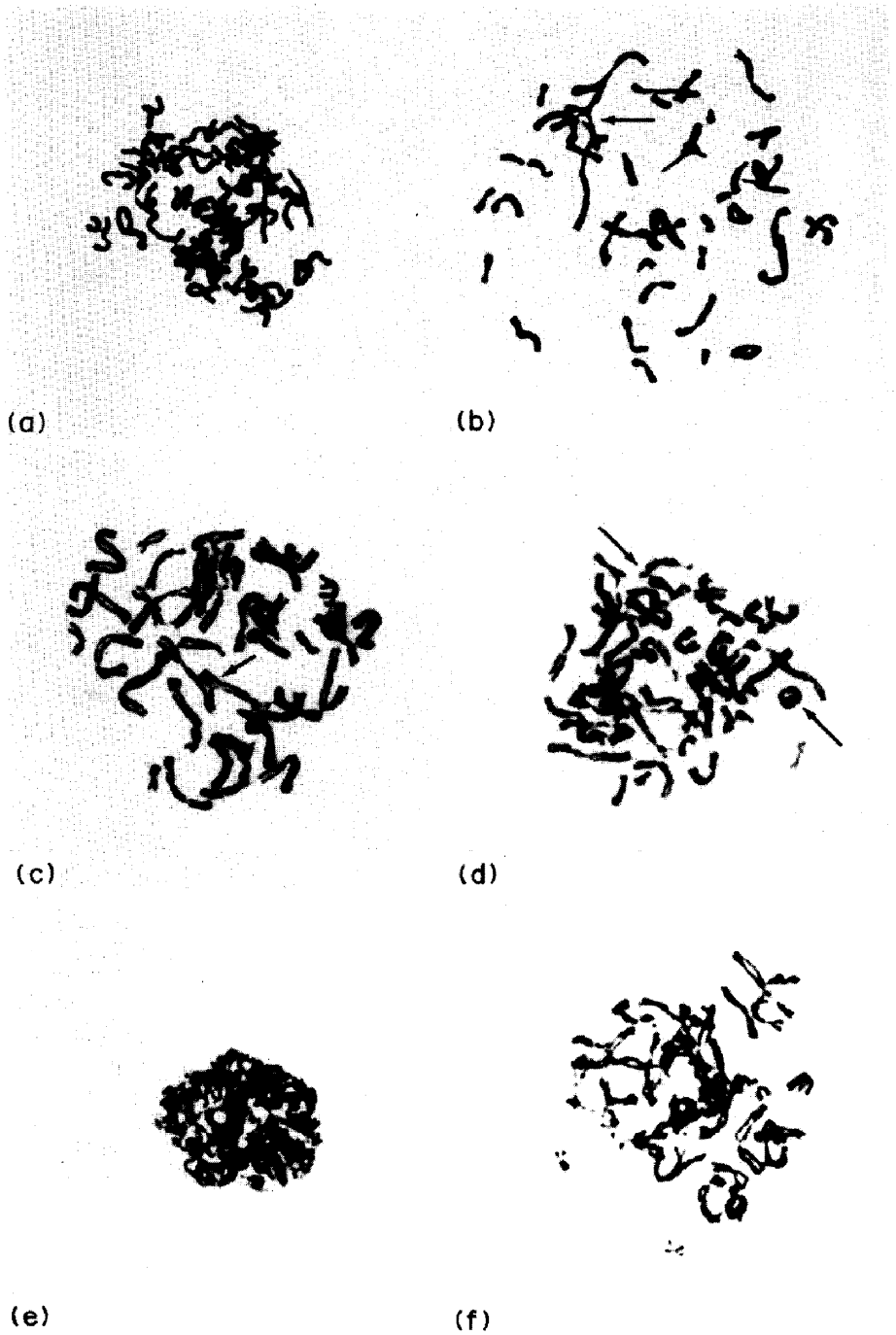


FIG. 2. Chromosome preparations of human PHA-stimulated lymphocytes incubated with 20 μg/ml of hycanthone. Hycanthone was added to lymphocyte cultures 4 hr before harvesting at 48 hr. Section (a) shows generalized chromosomal stickiness; arrows in sections (b) and (c) point to chromatid exchanges, and chromosomal elongation can also be seen; arrows in section (d) point to chromatid erosion and ring chromosome; section (e) shows chromosome stickiness; section (f) shows chromosome elongation, stickiness and fragmentation.

B.P.—*facing page* 1258

higher. Thus, at a hycanthone concentration of 20 μg/ml, the incidence of major chromosomal aberrations in cells examined in metaphase increased from the control level of 2 to 10 per cent. At hycanthone concentrations of 50 μg/ml, no chromosomal analysis could be made because of extensive damage to the chromosomes. The main chromosomal aberrations induced by hycanthone consisted of chromosomal elongation, stickiness, chromatid erosion, chromatid exchanges and breaks, and ring chromosomes. Representative examples of these hycanthone-induced aberrations are shown in Fig. 2.

Embryotoxicity of hycanthone. Table 4 shows that hycanthone administered on days 6–11 of gestation does not appear to possess significant teratogenic activity at any of the dose levels tested, although all doses of hycanthone resulted in fetuses with a significantly depressed body weight as compared to controls. Moreover, doses of 25 and 50 mg/kg increased the percentage of resorptions, and at the higher dose more than 90 per cent of the fetuses were killed.

Effects of hycanthone on DNA, RNA and protein synthesis. Figure 3 shows the effect of 20 μg/ml of hycanthone on DNA, RNA and protein synthesis of human lymphocytes at various intervals after the addition of PHA. Protein synthesis was not inhibited

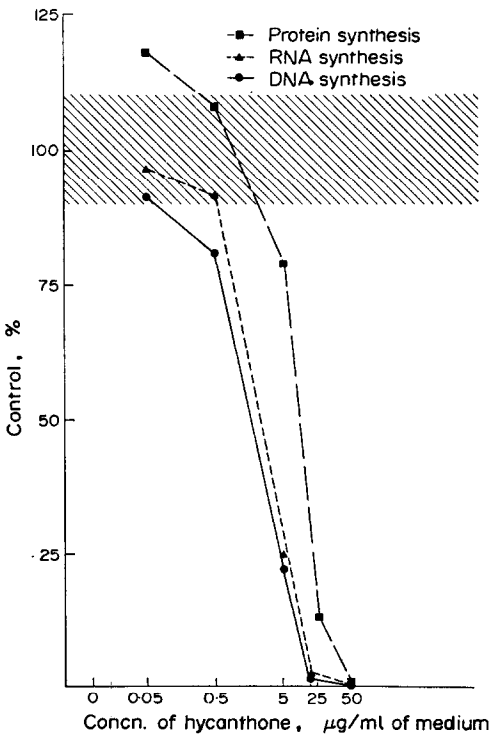


FIG. 4. Effect of various concentrations of hycanthone on DNA synthesis (●——●), RNA synthesis (▲——▲) and protein synthesis (■——■) of PHA-stimulated human lymphocytes. Hycanthone (0·05 to 50 μg/ml of medium) and PHA (0·1 mg/ml) were added at the beginning of the incubation. ^{14}C-2-uridine (0·5 μCi/ml) and ^{3}H-methyl-thymidine (0·5 μCi/ml) or ^{14}C-1-L-leucine (0·5 μCi/ml were added 2 hr before harvesting at 48 hr. Uptake of radioactivity into DNA, RNA and protein of cells incubated in the presence of hycanthone is expressed as per cent of uptake by control cells. The shaded area represents the range of control values.

at any time interval, whereas both DNA and RNA synthesis are inhibited at various time intervals with maximal inhibition occurring at 30 hr. The effects of varying concentrations of hycanthone on DNA, RNA and protein synthesis by PHA-stimulated human lymphocytes are shown in Fig. 4. At a concentration of 5 μg/ml, both DNA and RNA synthesis are inhibited 75 per cent, whereas inhibition of protein synthesis is minimal. At a hycanthone concentration of 50 μg/ml, macromolecular synthesis is totally inhibited.

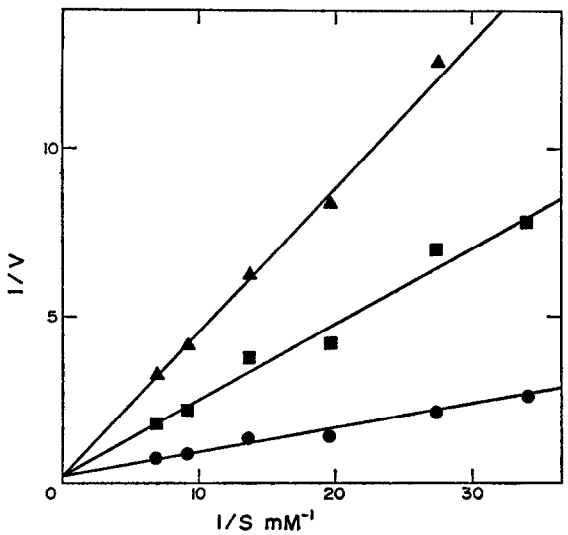


FIG. 5. Double reciprocal plot of inhibition of aldehyde oxidase (methotrexate oxidase) by hycanthone. Control cuvettes (●——●) contained: sodium phosphate buffer, pH 7·8, 120 μmoles; Versene Fe-3, 18 μg; rabbit liver aldehyde oxidase, 0·3 mg; and substrate (methotrexate) at the concentrations indicated, in a total volume of 3 ml. Assays were carried out at 37°. V = absorbance change per 10 min at 340 nm. Hycanthone concentration: ■——■, 17 μM; ▲——▲, 50 μM.

Inhibition of aldehyde oxidase. The enzyme aldehyde oxidase (EC 1.2.3.1) has been proposed as a convenient model for the study of inhibitors which affect enzyme-catalyzed electron transport.[19] Because of structural features in common with known *N*-alkylphenothiazine[22] and quinone[23] inhibitors of the enzyme, the activity of hycanthone as an aldehyde oxidase inhibitor was assessed. Figure 5 shows the effect of hycanthone at concentrations of 17 and 50 μM on the activity of aldehyde oxidase from rabbit liver. From the slopes of the double reciprocal plot shown, a K_i of 8×10^{-6} M was calculated for the inhibition of aldehyde oxidase by hycanthone.

DISCUSSION

Hycanthone is known to be an effective agent in the treatment of *S. mansoni* and *S. haematobium* infections.[1-3,24] In man, antischistosomal activity is observed after a single intramuscular injection of 3 mg/kg,[3] and this dosage regimen results in only a low degree of toxicity in animals and in man.[3,7,24] The mechanism by which hycanthone is able to exert a selective toxic effect against the schistosome while remaining a relatively nontoxic agent in the host is unknown.

The fact that hycanthone is mutagenic in an *Escherichia coli* bacteriophage system, in Salmonella[25] and in L5178Y mouse lymphoma cells[26] suggests that a site of action of hycanthone is at the level of DNA. Our experiments have shown that hycanthone inhibits the DNA synthesis of rapidly dividing PHA-stimulated lymphocytes. RNA synthesis was inhibited to approximately the same degree, whereas an inhibition of protein synthesis was observed only at higher hycanthone concentrations. It is interesting in this regard that Wittner *et al.*[27] found that hycanthone inhibits RNA synthesis in HeLa cells without affecting either DNA or protein synthesis.

Hycathone is a tricylic planar molecule containing a keto group and therefore possesses some chemical similarities to proflavine and actinomycin D, and other antitumor antibiotics. These compounds are known to inhibit macromolecular synthesis and it is thought that they do so through their ability to intercalate the base pairs of DNA.[28,29] There is some evidence that hycanthone may interact with DNA by intercalation,[30,31] which suggests that the mechanism of its inhibition of macromolecular synthesis may be similar to that of the acridines and actinomycin D.

The chromosomal aberrations induced in PHA-stimulated lymphocytes by hycanthone may well be the result of inhibition of DNA and RNA synthesis. A drug-induced depression of growth of rapidly dividing cells was manifested by the enbryotoxic activity of hycanthone in mice. Thus, approx. 90 per cent of conceptuses were killed with doses of hycanthone which were almost ten times smaller than the LD_{50} in mice.[32] These results are in agreement with those of Moore,[33] who found that hycanthone (50 mg/kg) killed 91 per cent of the fetuses of mice injected on days 7, 8 and 9 of gestation. Moreover, this author reported that a single injection of hycanthone at 35 or 50 mg/kg on day 7 of gestation resulted in a marked increase in the incidence of fetal malformations. The inhibitory effect of hycanthone on rapidly growing cells was again observed in tissue culture lines of leukemia L1210, Walker 256 carcinosarcoma and Novikoff hepatoma. Moreover, in systems *in vivo*, the activity of hycanthone against rapidly proliferating tissue was demonstrated by its ability to increase the median survival time of animals bearing five experimental tumors.

These results, along with earlier work reported by this laboratory,[34–36] suggest that hycanthone may have promise as an antitumor agent. Furthermore, our studies have shown that hycanthone is an inhibitor of the drug-metabolizing enzyme, aldehyde oxidase; in fact, it has greater inhibitory activity for this enzyme than do the *N*-alkylphenothiazines.[22] Aldehyde oxidase contains tightly bound iron, molybdenum and FAD as cofactors, and is susceptible to known inhibitors of electron transport such as amytal, antimycin A and oligomycin.[37] The physiological role of the enzyme is unknown, but it catalyzes the oxidative catabolism of a number of antitumor agents, including dichloromethotrexate,[38,39] procarbazine* and cyclophosphamide.[40] The antitumor activity of dichloromethotrexate has been shown to be potentiated by the simultaneous administration of phenothiazine inhibitors of aldehyde oxidase,[41] and it is possible that similar potentiation of the activity of antitumor agents metabolized by this enzyme may be obtained with hycanthone. Hycanthone has been reported to be hepatotoxic in two of eight patients treated for schistosomiasis,[42] and Cook and Jordan[43] reported a case of jaundice following treatment with this drug. Thus, in view of the toxic potential of hycanthone, its clinical trials in man must be carried out with

* R. H. Adamson, unpublished data.

caution. However, hycanthone does not appear to possess marrow toxicity, and because of its antitumor activity *per se*, and its inhibition of aldehyde oxidase, it may be especially valuable in the combination therapy of lymphomas.

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