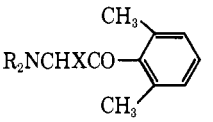
TABLE I
BIOLOGICAL RESULTS



No.	X	R	Potency ratio at 1 hr	Time (min) to 50% anesthesia at conen of 1%	0.5%	0.25%	Tissue damage
1	H (lidocaine)	C_2H_5	1	68	52	41	Slight
3	$CH_2CH{=}CH_2$	C_2H_5	1	>240	105	45	Severe at 1%
4	$CH_2C{\equiv}CH$	C_2H_5	3	205	180	160	Severe at all levels
2	CH_2SH	CH_3	0	27	0	0	Slight
8	$CH_2S-]_2$	CH_3	6	>240	>240	>240	Severe at all levels
5	CH_2SH	C_2H_5	0	0	0	0	Slight
9	$CH_2S-]_2$	C_2H_5	0	0	0	0	Slight
12	OOC_2H_5	C_2H_5	1	60	0	0	Slight
13	CH_2OH	C_2H_5	0.5	140	22	0	Slight
14	CH_2OOCH_3	C_2H_5	0.4	36	0	0	Slight
17	$CONH_2$	C_2H_5	0	0	0	0	Slight

(1%) (45–60 min). Compound **13** was active only at the 1% level and the effect was of shorter duration (30–60 min). In the irritancy test, 1% solutions of **13** and lidocaine gave no significant effects when compared with saline. Compound **3**, however, showed pronounced wheal formation at 1%, but was without effect at 0.5%.

In summary, therefore, of the ten compounds examined, four were without local anesthetic activity. Four compounds gave prolonged effects in the Bülbring and Wajda test; in two this was associated with irreversible tissue damage and in the nerve block test the duration of action of the other two did not differ markedly from that of lidocaine.

Acknowledgment.—We thank Mr. B. Basil for some of the pharmacological results and Mr. P. W. Tipton and Mrs. M. de Bruin for skillful technical assistance.

Hycanthone,[1] a New Active Metabolite of Lucanthone[2]

D. ROSI, G. PERUZZOTTI, E. W. DENNIS, D. A. BERBERIAN, H. FREELE, B. F. TULLAR, AND S. ARCHER

Sterling-Winthrop Research Institute, Rensselaer, New York

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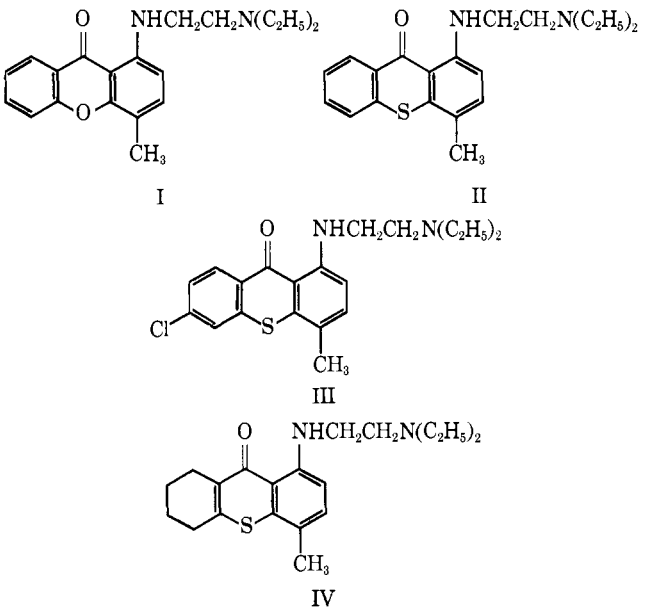
Microbiological oxidation of lucanthone[2] furnished hycanthone,[1] the 4-hydroxymethyl analog as the main product. Hycanthone is a highly active schistosomicidal agent when given orally or intraperitoneally. It has been identified chromatographically in the urines of man, monkey, and mouse following medication with lucanthone. Its chemical, physical, and biological properties suggest that it is the active metabolite of lucanthone.

Almost thirty years ago Mauss[3] synthesized a series of xanthenones, some of which were shown by Kikuth and Gönnert[4] to have schistosomicidal activity when administered orally to mice infected with a Liberian strain of *Schistosoma mansoni*. This was a signal chemotherapeutic achievement because this was the first orally effective nonmetallic organic compound found to possess such biological activity.

The most interesting members from the point of view of structure–activity relationships were miracil A (I), lucanthone (II), and the 6-chloro analog (III).

On the basis of evaluation in mice it was estabished that the thioxanthen-9-one II was more effective than



I II III IV

(1) For a preliminary communication see D. Rosi, G. Peruzzotti, E. W. Dennis, D. A. Berberian, H. Freele, and S. Archer, *Nature*, **208**, 1005 (1965). Hycanthone is the generic name for 1-[2-(diethylamino)ethylamino]-4-hydroxymethylthioxanthen-9-one.

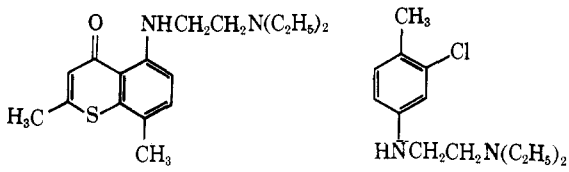
(2) Lucanthone is the generic name for miracil D.

(3) H. Mauss, *Chem. Ber.*, **81**, 19 (1948). Although the chemistry was reported after World War II, it is well known that the original work was carried out in the late 1930's.

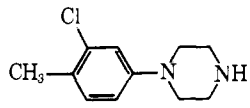
(4) W. Kikuth and R. Gönnert, *Ann. Trop. Med. Parasitol.*, **42**, 256 (1949).

the oxygen isostere I and that certain substituents (*e.g.*, chlorine) in position 6 of the thioxanthene nucleus increased activity. It was found and later confirmed by others[5] that the basic side chain can be varied over a limited range with little loss in biological activity. However, replacement of the 4-methyl group by other substituents abolished the schistosomicidal activity.[3-5] Reduction of the unsubstituted carbocyclic ring of II furnished IV which was an active schistosomicidal agent.[6] Further simplication of this ring system was achieved when it was discovered that the bicyclic thiochromone V was active.[7]

Mauss, *et al.*,[8] found that the monocyclic toluidine VI known as mirasan was highly effective in mice. The analogous piperazine VII was also reported to be active.[9]



V VI



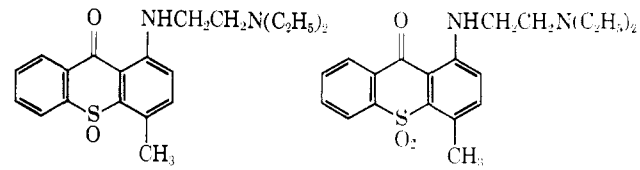
VII

The structural feature necessary for biological action common to all these classes of compounds is a dialkylaminoalkylamino group *para* to a methyl group on an aromatic ring. This conclusion is based on the schistosomicidal activity in mice. The results in *S. mansoni* infected monkeys are quite different. For example, III which is very effective in mice is essentially devoid of activity in the primate.[4] Mirasan VI is ineffective in monkeys and humans.[10] However, lucanthone, although only moderately effective in mice,[4] is highly effective in the monkey[4,10] and has enjoyed some clinical success[11] at doses which suggest that in man the drug is somewhat less active than in the monkey. Unfortunately the poor human tolerance of lucanthone militates against its being the drug of choice in the treatment of *S. haematobium* and *S. mansoni* infections.[11,12]

Biochemical and metabolic studies on lucanthone were soon initiated, owing to the clinical interest in the drug and to the suspicion, engendered in part by the host-dependent structure–activity relationships,[13] that the drug was being converted *in vivo* to the active agent. Although these investigations furnished little insight into the metabolic fate of the drug, more data were obtained to suggest that a metabolic transformation product was responsible for the observed schistosomicidal effect of lucanthone. The evidence may be summarized as follows: lucanthone is weakly active *in vitro*;[13] it is active in mice only when administered orally;[14] blood levels of lucanthone after oral administration are very low;[13] of seven species examined the most complex metabolic pattern is observed in the monkey, the one in which the drug is most effective;[10,15] and finally the host-dependent schistosomicidal activity discussed above.

The most recent and most extensive study of the metabolism of lucanthone was carried out by Strufe[15] who examined the urinary excretion products of lucanthone and its metabolites in several species including man, monkey, and mouse. The urines were collected and separated into neutral, acid, and basic fractions in such a way that at some point in the procedure exposure to acid occurred.

According to Strufe the major metabolites excreted by the mouse were the sufoxide VIII and the sulfone IX. Man excreted a "chromopeptide," the chromophore of which is the sulfoxide VIII. The monkey furnished a series of metabolites the most prominent of which again was the sulfoxide VIII. Other substances were not identified but the sulfone appeared to be absent in the urines of primates.



VIII IX

Microbial Metabolism.—Our approach to the problem of metabolism of lucanthone was begun from an entirely different vantage point. The compound was used as a substrate for microbiological exposure to a large number of different microorganisms. Of the organisms studied, *Aspergillus sclerotiorum* most efficiently converted the drug to a mixture of three new compounds which were readily separated by thin layer and column chromatography. These new metabolites retained the characteristic yellow color of the thioxanthen-9-one nucleus and were isolated in pure crystalline form. The elementary analysis of the most abundant member X (Chart I) of this mixture indicated that an oxygen atom was introduced into the molecule. The alcoholic nature of this new function was demonstrated by the easy preparation of the corresponding acetate XI. The nmr spectra of II, X (Figure 1), and XI clearly showed that the 4-methyl signal in the spectrum of lucanthone was replaced by a signal for a methylene group next to oxygen. In the

(5) S. Archer and C. M. Sutter, *J. Am. Chem. Soc.*, **74**, 4296 (1952).

(6) F. Bossert, H. Heinecker, and R. Gönnert, German Patent 1,024,980 (1958).

(7) F. Bossert and R. Gönnert, German Patent 954,599 (1956).

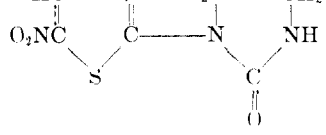
(8) H. Mauss, H. Kolling, and R. Gönnert, *Med. Chem., Abhandl. Med. Chem. Forschungsstaetten Farbwerke Hoechst A.G.*, **5**, 185 (1956).

(9) H. Ruschig, D. M. Schmidt, H. Leditschke, M. Schoor, and G. Lammler, German Patent 1,019,308 (1957).

(10) R. Gönnert, *Bull. World Health Organ.*, **25**, 702 (1961).

(11) D. M. Blair, *ibid.*, **18**, 989 (1958).

(12) A new antischistosomicidal agent which promises to be highly effective clinically is 1-(5-nitro-2-thiazolyl)-2-imidazolone: C. R. Lambert, *Experientia*, **20**, 452 (1964); this reference uses the code 32,644-Ba.

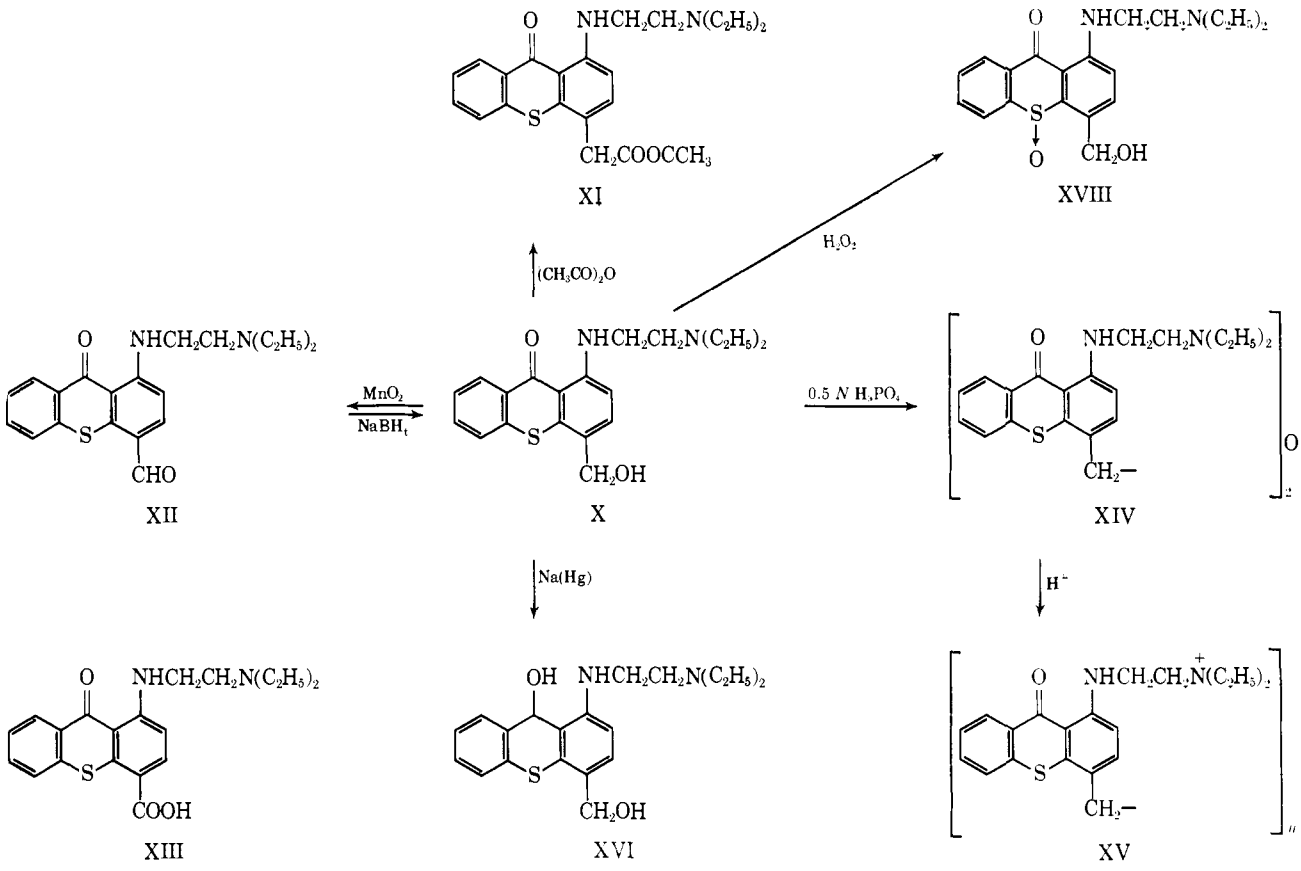


(13) (a) E. Buedding, A. Higashi, L. Peters, and I. F. Waite, *Proc. Soc. Exptl. Biol. Med.*, **64**, 110 (1947); (b) F. Hawking and W. F. Ross, *Brit. J. Pharmacol.*, **3**, 167 (1948); (c) I. Newsome and D. L. H. Robinson, *Trans. Royal. Soc. Trop. Med. Hyg.*, **54**, 582 (1960).

(14) D. A. Berberian and H. Freele, private communication.

(15) R. Strufe, *Med. Chem., Abhandl. Med. Chem. Forschungsstaetten Farbwerke Hoechst A.G.*, **7**, 337 (1963).

CHART I
HYCANTHONE AND SOME OF ITS TRANSFORMATION PRODUCTS



nmr spectrum of the acetate XI the signal at 309 cps was assigned to the methylene protons in the 4 position and the 123-cps singlet was assigned to the methyl of the acetate radical.

A chromatographically less polar companion of X was the aldehyde XII. The aldehydic nature of this compound was demonstrated by the elemental analysis, the presence of a carbonyl band in the infrared spectrum, and the nmr spectrum which showed a signal at 590 cps, characteristic of an aldehyde proton. Sodium borohydride reduction furnished the alcohol X[16] which was reoxidized to the aldehyde with MnO_2. The most polar microbial metabolite was the readily identified acid XIII. There was no indication that microbiological oxidation occurred on the sulfur atom of lucanthone. The sulfoxide XVII was obtained by H_2O_2 oxidation of X.

In the course of isolating the alcohol X from fermentation broths it was noted that the substance was quite sensitive to acid. Brief exposure of methylene chloride solution of X to the action of 1 *N* phosphoric acid served to convert the metabolite to a new high-melting substance. The elemental analysis and uv spectrum indicated little change in composition but there were no hydroxyl bands present in the spectrum of the new substance which was assigned structure XIV. Further exposure to acid of either X or XIV resulted in the formation of a water-soluble material showing a uv spectrum resembling that of the starting material. Since the new substance could not be removed from aqueous solution with an acidic Dowex ion-exchange column, it was felt that we were dealing with a polymeric quaternary ammonium salt as shown in structure XV. Reduction of X with sodium amalgam gave the thioxanthen-9-ol XVI.

Biologically the most outstanding property of X was its high oral schistosomicidal activity in mice and hamsters. In the former species X was about three to four times as active as II. In contrast to lucanthone the new hydroxymethyl compound was active when administered intraperitoneally. This new metabolite of lucanthone has been given the generic name hycanthone.

In view of the biological activity of hycanthone considerable effort was expended on a study of the fermentation procedure in an attempt to augment the yield of the over-all conversion of lucanthone to hycanthone, to increase the concentration of substrate, and to simplify the isolation procedure. It was found that careful control of the sterility and composition of the medium, pH of the fermentation, and rate of substrate addition served to increase the over-all conversion of II to X and increase the efficiency of the process.

One of the factors which limited high initial concentrations of the substrate was its inherent fungicidal activity. At levels of 1.5 g/l., growth inhibition of *A. sclerotiorum* occurred. Fortunately, hycanthone was much less potent in this respect and efficient conversions of substrate were achieved by the gradual addition of II to the fermentation medium at such a rate that sublethal concentrations were maintained at all times.

(16) This conversion proved to be a convenient preparative method for labeling X. The substitution of sodium borotritide in the reduction afforded the tritiated alcohol containing a tritium atom on the carbon in the hydroxymethyl group.

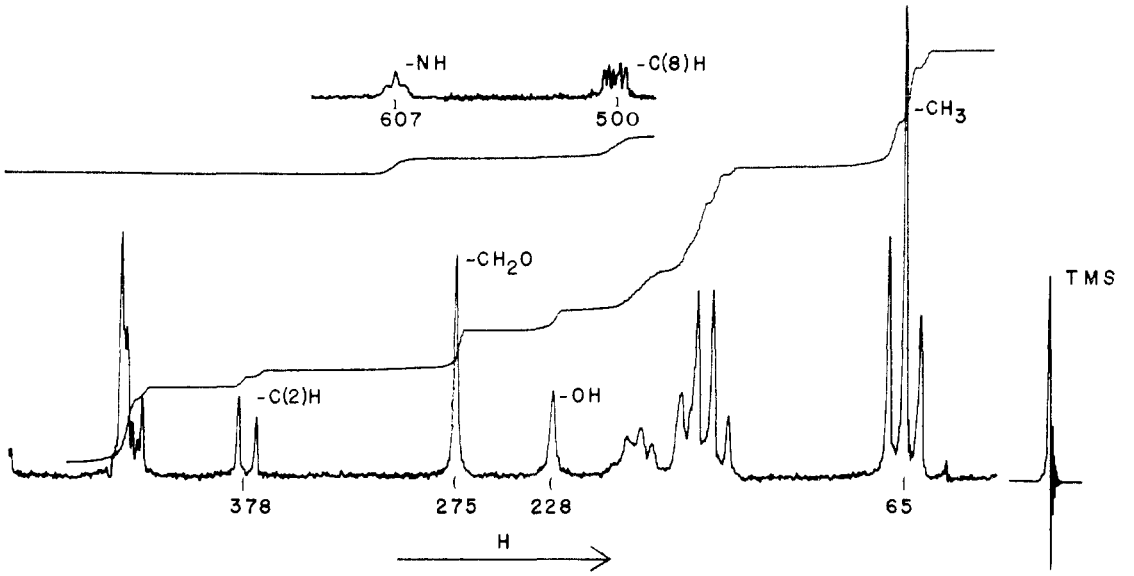


Figure 1.—Nmr spectrum of hycanthone (20% $CDCl_3$) with TMS as internal standard. Signals are given in Hz.

The isolation of the desired hydroxymethyl compound was greatly simplified by incorporating a $NaBH_4$ reduction in the isolation procedure. This maneuver served to convert the aldehyde XII, a troublesome impurity which comprised between 4–10% of the fermentation products, to the desired compound X.

By incorporation of these improvements the substrate concentration was increased from an initial value of 0.6 g/l. to at least 13.0 g/l. in 10-l. runs and yields of pure hycanthone of about 60% or more were realized without resorting to chromatography.

The conditions which were best for the fermentative oxidation of lucanthone did not necessarily apply to other related substrates. For example, the optimum pH for lucanthone conversion on a small scale is between 5.4 and 6.0; no reaction occurs above pH 7.0 in the first 24 hr. On the other hand the most suitable pH for the action of *A. sclerotiorum* on the N-deethyl derivative, 1-[2-(ethylamino)ethylamino]-4-methylthioxanthen-9-one (XIX), was 7.0 or over.

For screening purposes fermentations on xanthenes and thioxanthenes were generally carried out at about pH 6. In successful runs the yields of the corresponding hydroxymethyl metabolites ranged from 10 to 45% and they were usually accompanied by some of the corresponding 4-carboxaldehyde.

Occasionally, no suitable conditions were found for microbiological oxidations of thioxanthen-9-ones. Despite much experimentation no satisfactory conditions were found on a preparatory scale for converting the sulfoxide III to the hydroxymethyl analog XVII.

The main products of a number of fermentations are collected in Table I. These compounds were tested for their schistosomicidal activity in mice and hamsters according to the method of Berberian and Freele.[17]

The high schistosomicidal potency of hycanthone in the hamster is particularly noteworthy. It is about ten times more potent than lucanthone by oral administration and it is about five times more active when given intraperitoneally than when given orally. It is to be recalled that in our laboratory lucanthone is ineffective when given parenterally.[14]

A study of the biological results summarized in Table I permits a few conclusions to be drawn. Where direct comparisons have been made the hydroxymethyl derivative is usually more active than the corresponding 4-methyl compound. The optimum side chain in the 4-hydroxymethyl series seems to be diethylaminoethylamino. The others such as the piperidinoethylamino and monoalkylaminoethylamino are less effective. This is roughly parallel to the results noted previously in the 4-methyl series.[10] Another parallelism exists in the xanthene–thioxanthene pairs. The sulfur analogs are more active than their oxygen counterparts in both series. The tetrahydrothioxanthen-9-one IV is in reality a thiochromone. Its 4-hydroxymethyl analog is more potent than IV indicating that this parallelism is not confined to xanthenes and thioxanthenes.

A divergence is noted in the 6-chloro-substituted compounds. Kikuth and Gonnert[4] found that III is more potent than lucanthone in mice. However, in the 4-hydroxymethyl series this activity is reversed.

In general, in this series the schistosomicidal activities in the mouse are lower than in the hamster. This may be due to one or more of the following factors: poorer absorption of the drugs from the intestinal tract of the mouse, more rapid destruction of the drug in the stomach of the mouse, or more rapid hepatic metabolism in the mouse.

Could hycanthone qualify as the long-sought active metabolite of lucanthone? It was more potent than its 4-methyl analog. It was active parenterally and was also active *in vitro*[18] as the true drug should be. If the 4-methylthioxanthen-9-one had to be activated by metabolic oxidation of the 4-methyl group to a hydroxymethyl group then the experience with the 6-chloro compound III could be rationalized. The acid sensitivity could explain why X had escaped detection by previous investigators. On the basis of these considerations we were prompted to reinvestigate the mammalian metabolism of lucanthone.

Since lucanthone appears to be more effective in the monkey, an animal which appears to give the most

(17) D. A. Berberian and H. Freele, *J. Parasitol.*, **50**, 435 (1964).

(18) A. Yacinsky and D. A. Berberian, unpublished work.

complex urinary excretion pattern of metabolites, we decided to reinvestigate this species first. Accordingly, monkeys were medicated with lucanthone and the urines were collected and carefully extracted in such a manner that exposure to the action of acidic media was avoided. Examination of the extract by means of thin layer chromatography revealed the usual multiplicity of spots, the major one corresponded in R_f to the sulfoxide VIII. There was a faint spot corresponding to hycanthone. However, when the urines were first incubated with the enzyme glucuronidase and the extraction procedure repeated, the spot corresponding to hycanthone increased markedly in intensity. Apparently the new metabolite was excreted as a glucuronide, a not unexpected finding if indeed lucanthone was being transformed to hycanthone by the monkey. The ultraviolet spectrum of the new metabolite was the same as that of hycanthone and cochromatography with an authentic sample of X in several solvent systems indicated that the two were identical.

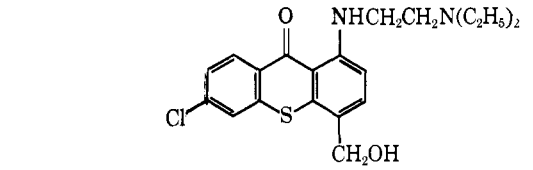
Lucanthone is generally thought to be more effective in the monkey than in the mouse. Its action in man probably occupies an intermediate position between these species. Although the intraspecies comparisons are difficult to make, owing to differences of degree of infection and to differences in criteria for cure, nevertheless, a reasonable estimate of species potency can be made. In this laboratory the ED_{50} (*po*) of lucanthone in mice is regarded to be 40–50 mg/kg/day given for 5 consecutive days. In the monkey doses of 10 mg/kg/day for 5 days can induce radical cures. The human dose is about 25 mg/kg/day for 3 or 4 days.[9] This regimen effects cures in greater than 50% of the patients receiving medication.

We have compared the urinary excretion patterns of these three species after medication with lucanthone. In contrast to Strufe we could not detect the sulfone IX[9] in the urine of mice. (Heterocyclic sulfides on metabolic oxidation generally furnished the corresponding sulfoxide but exceptions are known.[19]) Hycanthone could be detected only in trace amounts even after glucuronidase treatment. The finding is in keeping with the relatively poor activity of lucanthone in this species.

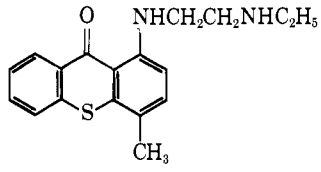
A volunteer took lucanthone and his 24-hr urine sample was examined for hycanthone. Our procedure did not permit us to detect Strufe's "chromopeptide," although the sulfoxide IX appeared to be present. Visual inspection of the thin layer chromatography of the urine extracts after glucuronidase treatment indicated that the amount of hycanthone present was greater than that noted in the mouse but less than that excreted by the monkey. Thus, the urinary excretion of hycanthone in these species is roughly parallel to the potency of lucanthone in the same species. These preliminary findings, which need confirmation by more quantitative work, are compatible with the hypothesis that lucanthone is metabolically converted to an active metabolite.

Kikuth and Gönnert[4] had found that in the mouse 6-chloro-1-[2-(diethylamino)ethyl]-4-methylthioxanthen-9-one (III) was more active than lucanthone but inactive in the monkey. If the biological activity of III depended on its metabolic conversion to XVIII then the latter ought to be readily detected in the urine of mice and to be absent in the urine of medicated monkeys.

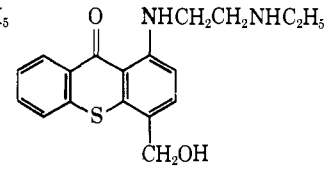
The hydroxymethyl derivative XVIII was obtained by incubation of III with *A. sclerotiorum* and served as the reference substance. Its structure was confirmed by nmr and uv spectroscopy. As in the case of hycanthone, the 6-chloro analog III was administered to monkeys and mice, and the urines were examined for the presence of the hydroxymethyl metabolite by means of thin layer chromatography and uv spectroscopy. XVIII was readily detected in the mouse urine but could not be found in the urinary extracts of the monkey.



XVIII



XIX



XX

The greater effectiveness of III in mice as compared to lucanthone could be due to either of these factors: (a) the drug is metabolized to the hydroxymethyl analog more efficiently then lucanthone in the mouse, or (b) XVIII is intrinsically more active than hycanthone. The last alternative was found to be incorrect by comparison of the ED_{50}'s of the two drugs (Table I).

In our view the experimental observations discussed above support the hypothesis that lucanthone and some of its congeners are indeed being converted to an active metabolite which is the corresponding 4-hydroxymethyl analog. On the basis of an analysis of structure–activity relationships Gönnert[10] concluded that in the lucanthone and related series a methyl group *para* to the basic side chain is essential for activity. The biological basis for this conclusion has now been established.

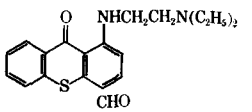
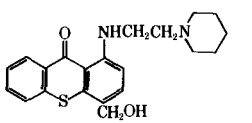
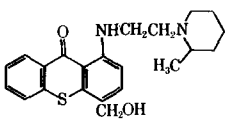
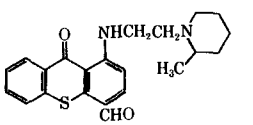
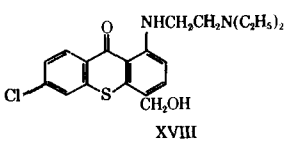
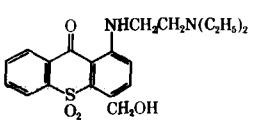
During the course of this study of lucanthone metabolism in the monkey we attempted to identify some of the other urinary excretion products. Owing to the complexity of the metabolism of this drug in the monkey[20] we confined our attention to those compounds which were, first, fairly mobile in a thin layer chromatography system in which the developing solution was ether–methanol–triethylamine (8:1:1); second, soluble in dilute mineral acid, indicating that the aliphatic amine residue was still present; and third, spectroscopically unchanged by the presence of alkali, an indication that no ring hydroxylation had occurred. In one monkey, fed a total of 156 mg of drug, only 5% could be accounted for as lucanthone or its transformation products. Of this about 60% was directly ex-

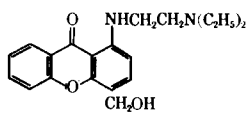
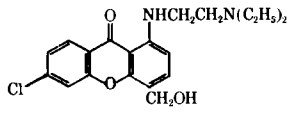
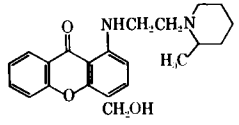
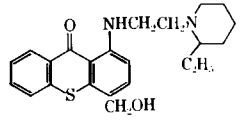
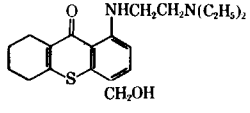
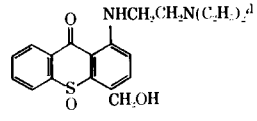
(19) R. T. Williams, "Detoxication Mechanisms," 2nd ed, John Wiley and Sons, Inc., New York, N. Y., 1959, p 663 ff.

(20) Visual inspection of a carefully prepared thin layer band chromatogram revealed at least twelve distinct and mobile bands with a great deal of material still present at the origin.

TABLE I

XANTHEN-9-ONES AND THIOXANTHEN-9-ONES BY *A. sclerotiorum* FERMENTATIONS

No.	Compound	Mp, °C (cor)	Formula	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N	$\lambda_{max}^{C_2H_5OH}$, mμ (ε × 10⁻³)	Schistosomicidal act. ED_{50}, mg/kg[a] *po*: 4-CH_2OH Mice	4-CH_2OH Hamsters	4-CH_3 Mice	4-CH_3 Hamsters
1	 X	100.6–102.8	$C_{20}H_{24}N_2O_2S$	67.38	6.79	7.86	67.20	6.49	7.96	233 (19.4) 258 (37.0) 329 (9.7) 428 (6.6)	14.2 ± 0.8	0.93 ± 0.1	46.0 ± 2.4	8.0 ± 1.0[c]
2	 XII	119.4–120.6	$C_{20}H_{22}N_2O_2S$	67.77	6.26	7.90	67.20	6.42	7.71	250 (27.6) 263 (16.3)[g] 298 (22.1) 330 (22.7) 411 (7.1)	>25	6.25 ± 3.2		
3		147.0–149.0	$C_{21}H_{24}N_2O_2S$	68.45	6.57	7.60	68.66	6.68	7.20	223 (19.0)[g] 234 (22.6) 258 (44.1) 332 (8.7) 441 (8.1)	27.3 ± 5.5	5.0 ± 1.6	70.5 ± 10.9	25.5 ± 5.5
4		112.6–113.8	$C_{22}H_{26}N_2O_2S$	69.08	6.85	7.33	69.25	6.92	7.21	235 (33.5) 258 (44.8) 334 (9.0) 443 (8.2)	22.3 ± 3.2	1.8 ± 0.5	50.2 ± 11.1	7.8 ± 1.3
5		131–134	$C_{22}H_{24}N_2O_2S$	69.44	6.36	7.36	69.30	6.36	7.23	250 (29.7) 263 (17.6)[g] 298 (23.9) 330 (24.2) 411 (7.9)	Not tested			
6	 XVIII	114.8–116.5	$C_{20}H_{23}ClN_2O_2S$	61.45	5.93	7.17	61.29	5.90	6.79	261 (49.0) 336 (8.6) 443 (7.7)	18.8 ± 4.8	6.0 ± 4.2	13.3 ± 3	>25
7		126.6–127.8	$C_{20}H_{24}N_2O_4S$	61.83	6.23	8.25[e]	62.12	6.42	8.11[e]	229 (22.7) 237 (32.7) 242 (21.2) 290 (7.0) 457 (8.5)	>12.5	Not tested	>12.5	>75

No.	Compound	Mp, °C (cor)	Formula	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N	$\lambda_{max}^{C_2H_5OH}$, mμ (ε × 10⁻³)	Schistosomicidal act., ED_{50}, mg/kg[a] *po* 4-CH_2OH Mice	4-CH_2OH Hamsters	4-CH_3 Mice	4-CH_3 Hamsters
8		131.0–132.5	$C_{20}H_{24}N_2O_3$	70.56	7.10	8.23	70.52	7.21	8.22	233 (41.3) 256 (24.3) 330 (5.75)[g] 311 (6.4) 417 (8.3)	25	10.6 ± 5.3	50	22.5 ± 10.9
9		130–132.5	$C_{20}H_{23}ClN_2O_3$	64.08	6.18	9.46[f]	64.01	6.40	9.49[f]	237 (39.5) 258 (19.3) 272 (20.1) 301 (6.7)[g] 310 (7.65) 422 (7.6)	Not tested	8.0 ± 2.0	Not tested	Not tested
10		134–136.2	$C_{22}H_{26}N_2O_3$	72.11	7.15	7.65	72.02	7.32	7.61	233 (42.9) 254 (24.0) 303 (5.7)[g] 310 (6.3) 418 (8.3)	61 ± 2.3	*Ca.* 12.5	Not tested	>25
11	 XX	150.0–151.6	$C_{18}H_{20}N_2O_2S$	65.83	6.14	9.72[e]	65.89	6.05	9.62[e]	234 (21.8) 258 (45.0) 330 (8.6)	19.5 ± 5.2	2.6 ± 0.8		
12		91–92	$C_{23}H_{28}N_2OS$	69.66	7.12	8.09	69.61	7.42	7.82[e]	235 (21.8) 258 (42.7) 332 (8.2) 443 (7.9)	23.0 ± 1.0	13.4 ± 2.3	21.5 ± 1.4	
13		125.6–127.2	$C_{20}H_{28}N_2O_2S$		8.90[e]	7.77		9.23[e]	7.73	229 (14.0) 251 (26.6) 304 (10.2) 327 (5.35) 414 (7.33)	>25	4.8 ± 1.9	>25	>50
14	 XVII	119–121	$C_{20}H_{24}N_2O_3S$	64.49	6.49	7.52	64.54	6.19	7.17	232 (28.9) 290 (6.0) 460 (8.7)	>12.5	3.1 ± 1.1	54 ± 14.7	>50

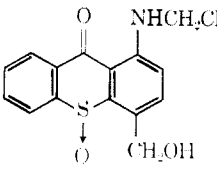
[a] The dose reported was given orally once a day for 5 days. Treatment was started 46 days post-infection. [b] Compounds labeled with Roman numerals are discussed in the text. [c] The intraperitoneal ED_{50} was 0.8 mg/kg given in one dose only. [d] This compound was obtained by direct oxidation of hycanthone. [e] S analysis. [f] Cl analysis. [g] Shoulder.

tractable into dichloromethane from the basified urines and the remaining was liberated by glucuronidase treatment.

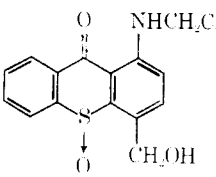
Due to the small amount of each metabolite that was excreted, these urinary excretion products were characterized on the basis of the following properties: (1) since the preliminary fractionation indicated that they were all moderately basic ($pK_a > 9.5$), the compounds were thought to be aliphatic amines; (2) since their uv spectra were not affected by the addition of alkali they were considered to be nonphenolic; (3) the substances that were directly extracted into dichloromethane were judged to possess a 4-methyl group; (4) those which were extractable after glucuronidase treatment were assumed to have a 4-hydroxymethyl group; (5) the substances which had λ_{max} at 285, 330, and 440 mμ were assigned the thioxanthen-9-one nucleus, whereas those displaying λ_{max} at 259 and 460 mμ were believed to possess the thioxanthen-9-one 10-oxide chromophore.

Finally, cochromatography with authentic specimens in tlc was carried out before the structures were assigned to the metabolites. Using these criteria five metabolites of lucanthone were identified with a fair degree of confidence and provisional assignments were made for two others.

The following monkey urinary excretion products in order of increasing polarity (tlc) were characterized. (1) The least polar substance was lucanthone itself. (2) The next was hycanthone X. After glucuronidase treatment it was extracted from urine at pH 9.5. Cochromatography with an authentic sample confirmed its identity. (3) The next substance was the most abundant. It was lucanthone sulfoxide (VIII) in agreement with Strufe's observations.[15] It represented about 30% of the total products, whereas hycanthone accounted for about 11%. The sulfoxide showed the characteristic λ_{max} 294, 470 mμ and also identical chromatographic behavior with an authentic sample prepared by hydrogen peroxide oxidation of lucanthone.[21] (4) The next most polar substance was the deethyl analog of lucanthone (XIX), identical in all respects (pK_a, uv spectrum, chromatographic behavior) with an authentic sample.[21] (5) The next compound was liberated after glucuronidase incubation and showed typical thioxanthen-9-one 10-oxide absorption in the ultraviolet. It was chromatographically identical with a sample prepared by oxidation of hycanthone with hydrogen peroxide. Accordingly, it was assigned structure XVII. (6) The next most polar compound resembled hycanthone in that it was liberated by glucuronidase treatment and had a similar uv spectrum and basicity. However, it was considerably more polar. These data suggested that it was a deethyl analog XX. An authentic sample was prepared by microbiological oxidation of XIX. The R_f of this microbial metabolite in the ether–methanol–triethylamine system was identical with that of the monkey metabolite. Both samples showed the same R_f in the ethyl acetate–triethylamine system. (7) The next compound was liberated by treatment with glucuronidase and the uv spectrum was characteristic of thioxanthen-9-one 10-oxides. Its low mobility and



XXI



XXII

basicity suggested that it was XXI, the sulfoxide of XX. Another relatively immobile substance had similar properties. It may be the primary amine XXII. However, in the absence of direct chromatographic comparisons with authentic specimens we regard the structural assignments of XXI and XXII as provisional only.

We have demonstrated that, at least in the thioxanthene series, it is not the 4-methyl group but the 4-hydroxymethyl group which is essential for schistosomicidal activity. In view of the fact that the 4-hydroxymethyl derivatives in the xanthene and thiochromone series are more active than the corresponding 4-methyl counterparts, it is highly likely that the 4-methyl groups in these systems are metabolically oxidized to the 4-hydroxymethyl groups. We have extended this investigation to the mirasans (VI and VII) and our results are reported in the following paper.[22]

Experimental Section

Analyses were carried out under the supervision of Mr. K. D. Fleischer. The nmr and ir spectra were carried out under the supervision of Dr. R. K. Kullnig, who also assisted in their interpretation. All nmr spectra were run on a Varian A-60 nmr spectrometer using TMS as the internal standard.

General Description of Microbiological Screening.—The microorganisms used were inoculated on Sabouraud's agar slants and incubated at 25° for 1 week. Ten milliliters of sterile distilled H_2O was added to the slant, and the spores and some vegetative growth was loosened and added to a 100-ml aliquot of a sterile medium of the following composition: 5 g of soybean meal, 20 g of dextrose, 5 g of NaCl, 5 g of K_2HPO_4, 5 g of yeast extract, 1 l. of tap H_2O, adjusted to pH 6.4. The flask containing the inoculum and the medium was agitated on a rotary shaker with a 1-in. throw at 240 rpm for 24 hr at 27°. This furnished the first-stage seed. To two flasks each containing 50 ml of the above-described medium was added a 10% inoculum of the first-stage seed and incubation on the rotary shaker was continued for 48–72 hr. Then 5 mg of substrate (*e.g.*, lucanthone) dissolved in a minimum quantity of aqueous ethanol was added to one of the flasks while the other served as a control. The incubation was allowed to proceed for another 24–48 hr. The contents of each flask were then extracted separately with dichloromethane, the solvents were evaporated, and the residues were taken up in 1 ml of the same solvent. About 25 μl of each was applied to a thin layer silica plate for chromatographic examination. In the case of lucanthone and its congeners the most satisfactory solvent system was ethyl acetate–triethylamine (9:1 v/v). A large number of microorganisms were tested using lucanthone as the substrate. Three metabolites were obtained, all of which were more polar than the original substrate. These were identified (see below and Table I) in order of polarity on the chromatogram as hycanthone (X), 4-carboxaldehyde (XII), and the 4-carboxylic acid (XIII). In general, *A. sclerotiorum* was the most satisfactory organism for converting 4-methylthioxanthen-9-ones and related structures to the corresponding hydroxymethyl compounds. Some of the transformation products are listed in Table I.

Conversion of Lucanthone to Hycanthone by *A. sclerotiorum* (10-l. Fermentation).—Several small-scale fermentations were

(21) We thank Mr. T. R. Lewis of this laboratory for carrying out this and some other chemical transformations reported here.

(22) D. Rosi, T. R. Lewis, R. Lorenz, H. Freele, D. A. Berberian, and S. Archer, *J. Med. Chem.*, **10**, 877 (1967).

carried out to determine optimum conditions. The effect of pH was studied in the following way. The screening procedure described above was modified so that 100 ml of pregrown cells were used along with 100 mg of substrate. The pH of the medium in several flasks was systematically adjusted from 4.5 to 7.5. After 24 hr of incubation at 27° aliquots from each of the 500-ml flasks were made alkaline and extracted with CH_2Cl_2. The hycanthone was separated from the lucanthone by means of thin layer chromatography; the compound was eluted from the plate and determined quantitatively by ultraviolet spectroscopy. Very little conversion occurred below pH 5.0 and above pH 6.5. Under these conditions maximum conversion occurred at pH 5.8: 100 mg of lucanthone furnished 37.5 mg of hycanthone. For longer fermentations (>24 hr) the optimum initial pH was 6.4, since the medium tended to become more acidic after 1 or 2 days.

In order to obtain some information about the upper limits of the initial substrate concentrations and the final permissible concentration of the major metabolite, the fungicidal activity with respect to *A. sclerotiorum* of lucanthone and hycanthone was determined. Complete inhibition of mycelial growth occurred when the level of the former reached 150 μg/ml. It was necessary to increase the concentration of hycanthone to 1000 μg/ml before a similar effect could be achieved. Accordingly, the lucanthone was added portionwise over the course of the fermentation to permit maximum conversion per run.

The carbohydrate, nitrogen, and mineral sources of the medium were extensively varied before the formula shown below was adapted for the 10-l. fermentation. The initial concentrations were 2% soybean meal, 6% dextrose, 0.5% NaCl, 0.5% yeast extract, 2% K_2HPO_4, and 0.02% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.4 with dilute HCl, and the whole was autoclaved at 1.05 kg/cm² for 35 min.

To this medium (9 l.) which was kept at 28–29°, aerated at a rate of 4 l./min, and stirred at 400 rpm, a 10% inoculum of *A. sclerotiorum* was added and after 1 day it was followed by 10 g of lucanthone hydrochloride dissolved in H_2O. After 18 hr another 10 g was added and 8 hr later another 10-g portion was added. The incubation was allowed to proceed overnight and in the morning of the third day 10 g more of lucanthone was added followed by another 15 g of substrate 8 hr later. A total of 55 g was added over a 3-day period.

The contents of the vessel were extracted with two 20-l. portions of CH_2Cl_2. The extracts were concentrated to dryness *in vacuo*. The oily residue was dissolved in 400 ml of methanol and 2 g of $NaBH_4$ was added. The mixture was stirred for 4 hr and the methanol was evaporated under vacuum. The residue was dissolved in 500 ml of benzene, and, after filtration, the benzene was washed with several 200-ml portions of H_2O. The benzene was stirred with Darco, filtered through Celite, and evaporated, leaving a thick oil which crystallized after trituration with 100 ml of ether. The hycanthone was collected and dried, 34.9 g, mp 101–102.5°.

In later runs, the initial dextrose concentration was increased to 8% and maintained at a high level by periodic addition of the sugar as required. In this way the fermentation was extended to 8 days and total conversion levels of 13.5 g/l. of lucanthone were achieved.

1-(2-Diethylaminoethyl)aminothioxanthen-9-one-4-carboxaldehyde (XII) from Lucanthone.—Before the conditions described above were found, a large number of experiments were carried out. In one of these, the medium consisted of 2% soybean meal, 2.0% dextrose, 0.3% brewers yeast, 0.5% NaCl, and 0.5% K_2HPO_4. Five grams of lucanthone was used as the substrate. After 24 hr a total of four such runs were combined and extracted (CH_2Cl_2) and processed as described above. The extracts were combined and evaporated leaving a thick gummy residue which was mixed with 125 g of silica gel (Davison grade 923 100–200 mesh) and slurried with 300 ml of hexane. The hexane was decanted and the residue was placed on a silica gel column (7 × 38 cm) and developed with a solvent consisting of 40% ethyl acetate, 57.5% hexane, and 2.5% triethylamine. The eluate was collected in 500 ml fractions. Fractions 11–23 contained unconverted lucanthone and the desired aldehyde (XII). Fractions 36–79 were combined and rechromatographed on another column. This time 1-l. fractions were collected. Fractions 5–21 furnished 7.0 g of almost pure hycanthone, mp 92.2–95.4°.

The original fractions 11–23 were combined and rechromatographed on a smaller (3.5 × 44 cm) silica gel column. The developing solvent was ethyl acetate–hexane–triethylamine (60:37.5:2.5) and was collected in 500-ml fractions. Fractions 3–7, containing the aldehyde, were combined and taken to dryness leaving a residue which was crystallized from ethyl acetate–hexane. There was obtained a total of 1.15 g (from 20 g of lucanthone), mp 119.4–120.6° (cor) (see Table I for analyses and uv spectral maxima).

The nmr spectra showed that the methyl signal of lucanthone was replaced by a new one-proton signal at 611 cps attributable to the aldehyde proton. The ir spectrum revealed the presence of a band at 6.0 μ corresponding to the carbonyl group.

"Hycanthone Ether" (XIV) from Hycanthone.—This substance was first encountered during some preliminary runs. A dichloromethane extract of a lucanthone fermentation which contained equal parts of hycanthone and lucanthone was shaken for a few minutes with 0.5 *N* H_3PO_4. The acid extract was brought to pH 7.0 and extracted (CH_2Cl_2). The residue was chromatographed on silica gel using ether containing 1% methanol and 1% triethylamine. The early fractions contained unchanged lucanthone but the later fractions furnished 0.65 g of yellow crystals of the ether XIV: mp 166–168° (cor); $\lambda_{max}^{dioxane}$ 224 mμ (ε 29,900), 237 (30,400), 259 (44,000), 338 (10,800), 443 (8950). The R_f in the ethyl acetate–triethylamine system was 0.56, that for hycanthone was 0.36 and for lucanthone, 0.62.

Anal. Calcd for $C_{40}H_{46}N_4O_3S_2$: N, 8.06; S, 9.22. Found: N, 8.20; S, 9.09.

1-(2-Diethylaminoethyl)aminothioxanthen-9-one-4-carboxylic Acid (XIII) from Lucanthone.—In order to isolate the acid, two 10-l. fermentations in which a total of 45 g of lucanthone was added were extracted at pH 6.5. The CH_2Cl_2 extracts were worked up in the usual way but when benzene was added an insoluble substance separated which appeared to be the sodium salt of the desired acid (XIII). The material was taken up in warm dilute acetic acid to convert it to the free acid and then the solid was recrystallized from 95% ethanol to afford 1.0 g of yellow crystals, mp 128–131° (cor).

Anal. Calcd for $C_{20}H_{22}N_2O_3S$: C, 64.85; H, 5.96; S, 8.16; neut equiv, 370.4. Found: C, 64.72; H, 6.07; S, 8.18; neut equiv, 374.9.

The compound was inactive as a schistosomicidal agent.

1-(2-Diethylaminoethyl)aminothioxanthen-9-one-4-carboxaldehyde (XII) from Hycanthone.—To a dried suspension of active MnO_2[23] in 5 l. of benzene there was added 64 g of hycanthone and the mixture was stirred under reflux for 6.5 hr. After cooling, the whole was filtered to remove the inorganic material. The filtrate was concentrated to dryness and the residue was crystallized from 100 ml of isopropyl acetate to afford 36 g of the aldehyde in two crops, mp 113–117° (uncor). These were combined and recrystallized to give 30.6 g (48%) of the pure aldehyde, mp 119.4–120.6° (cor), identical in all respects with the material isolated from the fermentation procedure (Table I).

1-[2-(Diethylaminoethyl)amino]-4-acetoxymethylthioxanthen-9-one (XI).—Six milliliters of acetic anhydride was added to a solution of 10 g of hycanthone in 15 ml of pyridine and the whole was heated on the steam bath for 10 min. On cooling, crystals separated. These were collected, washed with hexane, and recrystallized from 275 ml of acetone to give 9.06 g of the acetate ester: mp 120.6–122.0° (cor); $\lambda_{max}^{95\% \text{ ethanol}}$ 237 mμ (ε 24,600), 258 (45,100), 329 (8050), 436 (8500).

Anal. Calcd for $C_{22}H_{26}N_2O_3S$: C, 66.30; H, 6.57; N, 7.03. Found: C, 66.36; H, 6.83; N, 7.06.

1-[2-(Diethylaminoethyl)amino]-4-hydroxymethylthioxanthen-9-ol (XVI).—One gram of the carboxaldehyde (XII) was dissolved in 175 ml of methanol and then 0.2 g of $NaBH_4$ was added. The yellow solution turned orange almost immediately and thin layer chromatography showed that the substrate had disappeared very rapidly. The resulting hycanthone solution was treated with 2.0 g of NaHg and then stirred at room temperature for 5 hr at which time the color had been discharged. The solution was diluted with 200 ml of ice–water and left overnight. The white

(23) The MnO_2 was prepared as follows. A solution of 240 g of $KMnO_4$ in 3 l. of water was stirred at 55°. Then 0.4 ml of 35% NaOH solution was added. Over the course of the next hour 150 ml of methanol was added dropwise. The suspension was heated at 95° for 1 hr and carefully neutralized by the slow addition of a solution of 35 ml of H_2SO_4 in 100 ml of water. The precipitate was collected and pressed as dry as possible. The moist cake was suspended in benzene and dried azeotropically with the aid of a Dean–Stark trap. R. J. Gritter, G. D. Dupre, and T. J. Wallace, *Nature*, **202**, 179 (1964), review briefly the preparation of MnO_2 suitable for this type of oxidation.

solid which separated was harvested and crystallized from ethyl acetate to give 0.289 g of the thioxanthen-9-ol: mp 132.0–133.0° (cor); $\lambda_{max}^{95\% \text{ EtOH}}$ 243 mμ (ε 19,300), 317 (4900). The nmr spectrum in heptadeuteriodimethylformamide showed a signal at 281 cps for two protons characteristic of the 4-methylene protons adjacent to the hydroxyl group.

Anal. Calcd for $C_{20}H_{26}N_2O_2S$: N, 7.81; S, 8.94. Found: N, 7.89; S, 9.03.

1-[2-(Diethylaminoethyl)amino]-4-hydroxymethylthioxanthen-9-one 10-Oxide (XVII).—A solution of 6 g of hycanthone in a minimum volume of 5% acetic acid was treated in the cold with 30 ml of 30% H_2O_2 and then left at 4° for 72 hr. The mixture was rendered alkaline with NH_4OH and then extracted with three 200-ml portions of CH_2Cl_2. The extract was concentrated and the residue was chromatographed on silica gel. The column was developed with methanol–ether containing 1% triethylamine in which the methanol concentration was increased. The methanol-rich fractions furnished 0.56 g of the desired sulfoxide as reddish crystals, mp 119–121° (cor) (Table I).

Mammalian Metabolism of Thioxanthen-9-ones. I. Lucanthone. A. Monkey.—Two rhesus monkeys were medicated (orally) with 150 and 158 mg, respectively, of lucanthone and the urines were collected in two portions: one for the first 24 hr and then for the second day. Portions from each collection period were adjusted to pH 9.5 and then extracted (CH_2Cl_2). Duplicate urine aliquots were adjusted to pH 6.2 with a phosphate buffer and incubated at 37° for 24 hr with 6000 units of β-glucuronidase after a few drops of toluene was added. The pH of these urine samples was then adjusted to 9.5 and extracted (CH_2Cl_2). This latter extract was referred to as "total drug excreted" in contrast to the first extract which represented the "nonconjugated bases." In order to obtain rough estimates of the amount of thioxanthen-9-ones that were excreted the optical density (OD) at 258 mμ was measured. The conversion factor was 1 μg/ml ≡ 0.120 OD at 258 mμ. Table II summarizes these results.

In the case of the first monkey about 5% of the total dose was recovered from the urine. Of this about two-fifths was excreted as glucuronic acid conjugates. In the second monkey only about 3.5% was recovered. About 30% of this was conjugated.

An estimate of the relative amounts of lucanthone, hycanthone, and lucanthone sulfoxide was made. Aliquots from the urine which were treated with glucuronidase were chromatographed on a silica gel plate and the components of the mixture corresponding in mobility to the thioxanthen-9-ones mentioned were eluted and an assay by uv spectroscopy was carried out using the same factor as above for lucanthone and hycanthone and adsorption at 294 mμ (1 μg/ml ≡ OD of 0.018) for the sulfoxide. The results for the first monkey are summarized in Table III.

Of the total recovered (8.25 mg) lucanthone represented about 4%, hycanthone about 11%, and the sulfoxide accounted for 30%.

TABLE II

Monkey	Period, hr	Nonconjugated, mg	Total, mg
1	0–24	2.5	4.25
	24–28	2.5	4.0
	Total	5.0	8.25
2	0–24	0.9	1.6
	24–48	2.8	3.7
	Total	3.7	5.3

TABLE III

Period, hr	Lucanthone, mg	Hycanthone, mg	Sulfoxide, mg
0–24	0.15	0.54	1.3
24–48	0.15	0.38	1.2
Total	0.30	0.92	2.5

Thus, the sulfoxide is the most abundant metabolite even after liberation of the hycanthone.

In order to separate and identify the metabolites, aliquots of "total drug excreted" were subjected to tlc using the following mixtures for development: ethyl acetate–triethylamine (system A) and ether–methanol–triethylamine (system B). Eight distinct metabolites (including hycanthone) were resolved. These were compared, wherever possible, with authentic samples, by tlc in systems A and B, and by uv spectroscopy. These comparisons are tabulated in Table IV. Finally, a portion of the "total drug excreted" was applied to the lower right hand corner of a 20 × 20 cm silica gel plate. After development in system B authentic samples of II, X, VIII, XIX, XVII, and XX were coapplied to the separated components and then the plate was redeveloped at right angles. On redevelopment in system B, the respective coapplied spots each remained as one component.

TABLE IV

COMPARISON OF MONKEY METABOLITES WITH AUTHENTIC SAMPLES

Compd	R_f System A	R_f System B	Uv, mμ
Metabolite 1	0.69	0.91	258, 330, 440
Lucanthone	0.69	0.91	258, 330, 440
Metabolite 2	0.33	0.69	258, 330, 440
Hycanthone	0.33	0.69	258, 330, 440
Metabolite 3	0.40	0.60	294, 470
Lucanthone sulfoxide (VIII)	0.40	0.60	294, 470
Metabolite 4	0.33	0.49	258, 330, 446
"Deethyl lucanthone" (XIX)	0.31	0.48	258, 330, 446
Metabolite 5	0.12	0.24	294, 460
Hycanthone sulfoxide (XVII)	0.12	0.34	290, 455
Metabolite 6	Unresolved	0.24	257, 330, 440
Deethyl hycanthone (XX)	0.07	0.24	257, 330, 440
Metabolite 7	0.08	0.17	292, 465
Metabolite 8	0.00	0.07	295, 455

B. Human.—Urine from a human subject who had taken 100 mg of lucanthone was collected for 24 hr. The urines were worked up as previously described in the monkey experiments. The major metabolites were hycanthone and the sulfoxide VIII (R_f system A was 0.33 and 0.40, respectively, identical with authentic samples applied for comparison). The total amount excreted represented about 1% of the ingested drug.

C. Mouse.—Urine from 100 mice medicated with 500 mg/kg of lucanthone was collected for 24 hr postmedication. Only traces of material corresponding in R_f to hycanthone was present. The major metabolite was a substance of R_f 0.08 in the ethyl acetate–triethylamine system that was orange rather than yellow in color, suggesting that oxidation of the sulfur atom had occurred. It was *not* lucanthone sulfone which has an R_f 0.54 in this system.

II. 1-[2-(Diethylaminoethyl)amino]-6-chloro-4-methylthioxanthen-9-one (III). A. Monkey.—Two monkeys were medicated with III (60 mg/kg *po*) and the urines were collected for 24 hr. They were subjected to enzymatic hydrolysis with β-glucuronidase before extraction with CH_2Cl_2 at pH 9.5. The extracts were examined by tlc using system A. In each case trace amounts of the original drug (R_f 0.70) was observed but the corresponding hydroxymethyl compound XVIII (R_f 0.39) could not be detected.

B. Mouse.—One hundred mice were medicated with III (500 mg/kg) in the same manner described above for lucanthone. Examination of the extracts by tlc revealed a component whose chromatographic and spectroscopic properties were the same as that of the authentic hydroxymethyl compound XVIII.