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Repair *in Vivo* of Liver Deoxyribonucleic Acid Damaged by Hycanthone and Related Compounds

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SUMMARY

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Liver DNA damage and its subsequent repair *in vivo*, induced by the antischistosomal drug hycanthone methanesulfonate and its analogues, was studied by sedimenting DNA in alkaline and neutral sucrose gradients. Within 1 hr after administration to rats, hycanthone methanesulfonate (25 μg/g of body weight) caused liver DNA damage which could be monitored as slowly sedimenting DNA (compared with control) in alkaline as well as neutral sucrose gradients. By 24 hr the hepatic DNA of rats given hycanthone (100 μg/g of body weight) sedimented into the 2.3 M sucrose layer of the neutral sucrose gradients, suggesting repair of the DNA lesions. However, when the same DNA was sedimented in alkaline sucrose gradients, anomalies were observed even 48 hr after administration of the drug. At the same dosage, two analogues of hycanthone, the chloroindazole derivative and its *N*-oxide, which are less hepatotoxic, teratogenic, and mutagenic than hycanthone, failed to induce the DNA damage measurable as slowly sedimenting DNA in neutral sucrose gradients. Nonetheless, these two analogues, like hycanthone, intercalated into DNA. Niridazole (200 μg/g of body weight), another antischistosomal drug, neither intercalated into DNA nor induced hepatic DNA damage measurable as slowly sedimenting DNA in either neutral or alkaline sucrose gradients.

INTRODUCTION

Hycanthone, 1-{[2-(diethylamino)ethyl]amino}-4-(hydroxymethyl)thioxanthen-9-one, is used in the treatment of schistosomiasis (1-3). Besides inducing chromosomal abnormalities (4-6), the drug has been shown to be mutagenic in mammalian cells (7) and carcinogenic in mice with schistosomiasis (8). Recently, using sedimentation analysis of DNA in alkaline sucrose gradients similar to that described by McGrath and Williams for bacteria (9), it has been shown that chemical carcinogens induce strand breaks in rat liver DNA *in vivo* (10-21). It was of interest to determine whether hycanthone and some other antischistosomal drugs, such as the chloroindazole derivative of hycanthone (IA4), its *N*-oxide, and niridazole, which are less hepatotoxic and mutagenic than hycanthone (22-27), would induce similar damage in liver DNA *in vivo*.

The results indicate that hycanthone

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when administered to rats causes liver DNA damage, which could be monitored as slow sedimentation of the DNA (compared with the control) in alkaline and neutral sucrose gradients. Some of the drug-induced DNA lesions, monitored as slow sedimentation of DNA in neutral sucrose gradients, were repaired faster than other damage in the same DNA, which could be monitored as slow sedimentation of DNA in alkaline sucrose gradients. In contrast, the antischistosomal drugs IA4, IA4-*N*-oxide, and niridazole did not induce liver DNA damage which could be monitored in neutral sucrose gradients.

MATERIALS AND METHODS

White male Wistar rats (Carworth Farms) weighing 100 g were partially hepatectomized in accordance with the procedure of Higgins and Anderson (28). Hycanthone methanesulfonate and hycanthone succinate were gifts from Dr. A. E. Farah, Sterling-Winthrop, and 8-chloro-2-[2-(diethylamino)ethyl]-2*H*-[1]-benzothiopyrano-[4,3,2-*cd*]-indazole-5-methanol monomethanesulfonate (IA4), an analogue of hycanthone, IA4-*N*-oxide, and niridazole were obtained from Dr. E. Bueding, Johns Hopkins University School of Hygiene and Public Health. Methanesulfonate (sodium salt) was obtained from Eastman Kodak. Figure 1 shows the structures of hycanthone, IA4, and IA4-*N*-oxide.

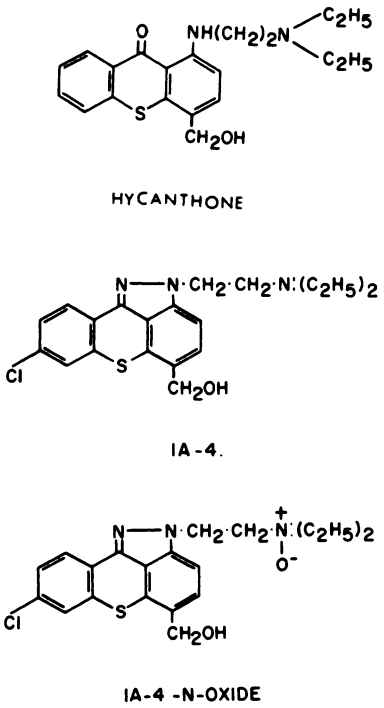

FIG. 1. *Chemical structures of hycanthone, IA4, and IA4-N-oxide*

Liver DNA was labeled with [*methyl*-^{3}H]thymidine (New England Nuclear) during liver regeneration as described earlier (12). The animals were used after a recovery period of 2 weeks. Rats were killed by decapitation at different time intervals following the intraperitoneal administration of 0.9% NaCl or 0.9% NaCl containing hycanthone derivatives or sodium methanesulfonate. Preparation of liver nuclear suspensions, alkaline and neutral sucrose gradients, lysis of nuclei, release of DNA on top of the gradient, and other technical details have been described previously (12, 21). The alkaline lysing solution consisted of 0.3 M NaCl, 0.03 M EDTA, 0.1 M Tris (pH 12.5), and 0.5% sodium dodecyl sulfate, and the neutral lysing solution was identical with the alkaline lysing solution, except that the pH was 7.5. Lysing of nuclei was carried out for 30 min at room temperature. Alkaline sucrose gradients were calibrated using DNA from T4 and T7 phages and nicked SV40. Molecular weight values higher than that of T4 phage DNA (the molecular weight of single-stranded T4 DNA is 6×10^7) were extrapolated from the sedimentation patterns of DNA from SV40 (nicked circles) and T7 and T4 phages (29).

Thermal denaturation of DNA. For measuring thermal denaturation, liver DNA was prepared by the procedure of Marmur (30) from rats treated with either 0.9% NaCl or hycanthone methanesulfonate (150 μg/g of body weight) for 4 hr. The purified rat liver DNA or calf thymus DNA (Sigma) was dialyzed overnight against 2 liters of 0.15 M NaCl and 0.015 M sodium citrate, pH 7.5, or 0.0015 M NaCl and 0.00015 M sodium citrate, pH 7.5. DNA was used at a concentration of 20 μg/ml. The temperature of the cuvette chamber

was increased at a rate of 0.2°/min, using a programmed Heto water bath (London Company, Cleveland, Oh.). The temperature of the cuvette chamber and the absorbance at 260 nm were recorded every 5 sec automatically, using a 2400S Gilford spectrophotometer.

RESULTS

Figure 2 shows typical sedimentation patterns of hepatic DNA from a control rat and from one treated with hycanthone methanesulfonate. The DNA from the control rat liver sedimented near the bottom of the gradient into the 2.3 M sucrose layer. Acid-precipitable radioactivity of gradient fractions was essentially free of protein and RNA (12) and was alkali (0.3 N NaOH)-stable, Pronase-insensitive, and DNase-sensitive, and hence represented DNA. This DNA was single-stranded, as judged by physical and electron microscopic methods, as well as by its susceptibility toward S_1 nuclease (29). Hepatic DNA of rats treated with hycanthone methanesulfonate (100 μg/g) for 60 min exhibited slower sedimentation characteristics than the control DNA in an alkaline sucrose gradient. Slow sedimentation of liver DNA was also seen with doses of 25 and 50 μg/g (Table 1). Doses of 25 and 50 μg/g exerted an identical effect on liver DNA; the reason for this lack of dose response is not clear. Nevertheless, a dose of 100 μg/g exerted a greater effect than both 25 and 50 μg/g.

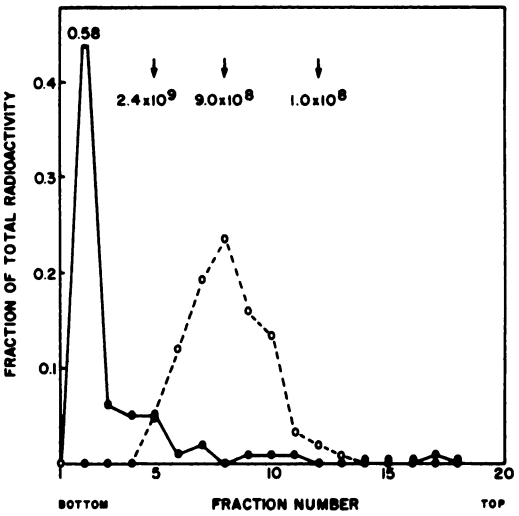

FIG. 2. *Sedimentation patterns in alkaline sucrose gradients of hepatic DNA from a control and a hycanthone methanesulfonate-treated rat*

Rats were given an intraperitoneal injection of either 0.9% NaCl (●——●) or 0.9% NaCl containing hycanthone methanesulfonate (100 μg/g) (O- - -O). Both rats were killed 1 hr later. Each gradient represents one animal. The acid-precipitable radioactivity recovered from each gradient was as follows: control, 800 cpm; experimental, 1100 cpm.

Table 2 shows data on sedimentation characteristics of hepatic DNA in alkaline sucrose gradients at different time intervals following the administration of hycanthone. At a dose of 100 μg/g, slow sedimentation of liver DNA was seen within 30 min, and by 24 hr a considerable portion of the DNA had become heavy, suggesting

TABLE 1

Sedimentation analysis in alkaline sucrose gradients of hepatic DNA following administration of different doses of hycanthone methanesulfonate

Hycanthone methanesulfonate in water or 0.9% NaCl or an equivalent volume of the carrier was given intraperitoneally. Rats were killed 1 hr later. Values are the means ± standard errors for the number of rats shown.

Dose of hycanthone methanesulfonate	No. of rats	Distribution of liver DNA in alkaline sucrose gradients			
		1–3 (>3 × 10⁹)[a]	4–8 (6 × 10⁸–3 × 10⁹)	9–14 (7 × 10⁶–6 × 10⁸)	15–18 (<7 × 10⁶)
μg/g body weight		*% total radioactivity*			
0	12	65 ± 2	17 ± 2	6 ± 1	3 ± 1
25	4	45 ± 6	36 ± 4	14 ± 4	2 ± 0.1
50	4	45 ± 3	40 ± 3	9 ± 2	1 ± 1
100	14	13 ± 4	57 ± 2	21 ± 2	3 ± 1

[a] Fraction and corresponding molecular weight range (in parentheses).

repair of the damaged DNA. Although repair was progressive with time, damage was persistent even at 48 hr. In neutral sucrose gradients, the slow sedimentation of liver DNA observed at 30 min after the administration of hycanthone methanesulfonate (100 μg/g, Table 3) persisted up to 4 hr, but by 24 hr the liver DNA had apparently become heavy (sedimenting in the 2.3 M sucrose layer). These sedimentation characteristics in neutral and alkaline sucrose gradients indicate that lesions detectable in the neutral sucrose gradients were repaired faster than those that could be monitored in alkaline sucrose gradients.

Hycanthone succinate (100 μg/g of body weight, dissolved in dimethyl sulfoxide) given intraperitoneally induced slow sedimentation of liver DNA both in alkaline and in neutral sucrose gradients by 1 hr. In one experiment, methanesulfonate (sodium salt, 100 μg/g of body weight) did not induce any obvious change in liver DNA (as measured by sedimentation analysis in neutral and alkaline sucrose gradients) either 1 or 4 hr after the administration of the compound.

In two experiments, IA4 (100 μg/g of body weight) induced slow sedimentation of liver DNA in alkaline sucrose gradients 24 hr after administration. However, unlike hycanthone methanesulfonate or hycanthone succinate, this dose of IA4 did not cause slow sedimentation of liver DNA in neutral sucrose gradients 1, 4, or 24 hr

TABLE 2

Sedimentation analysis in alkaline sucrose gradients of hepatic DNA at various times following administration of hycanthone methanesulfonate

Values are the means ± standard errors for the number of rats shown.

Drug	Time of death	No. of rats	Distribution of liver DNA in alkaline sucrose gradients			
			1-3 (>3 × 10⁹)[a]	4-8 (6 × 10⁸-3 × 10⁹)	9-14 (7 × 10⁶-6 × 10⁸)	15-18 (<7 × 10⁶)
			% total radioactivity			
None[b]		12	65 ± 2	17 ± 2	6 ± 1	3 ± 1
Hycanthone methanesulfonate (100 μg/g)	30 min	4	16 ± 10	50 ± 11	23 ± 5	5 ± 1
	60 min[b]	14	13 ± 4	57 ± 2	21 ± 2	3 ± 1
	4 hr	8	15 ± 6	58 ± 6	18 ± 3	5 ± 1
	24 hr	6	31 ± 5	53 ± 4	10 ± 2	2 ± 1
	48 hr	3	53 ± 2	29 ± 2	9 ± 2	1 ± 0.5

[a] Fraction and corresponding molecular weight range (in parentheses).

[b] The 1-hr values for controls and the group that received hycanthone methanesulfonate (100 μg/g) have been reproduced from Table 1 for easy reference.

TABLE 3

Sedimentation analysis in neutral sucrose gradients of hepatic DNA following administration of single doses of hycanthone methanesulfonate or IA4

Values are the means ± standard errors for the number of rats shown.

Drug	Time of death	No. of rats	Distribution of liver DNA in neutral sucrose gradient			
			1-3[a]	4-8	9-14	15-18
			% total radioactivity			
None		6	73 ± 5	14 ± 1	8 ± 2	7 ± 1
Hycanthone methanesulfonate (100 μg/g)	30 min	7	16 ± 6	47 ± 11	25 ± 11	9 ± 3
	60 min	7	9 ± 2	61 ± 8	15 ± 7	10 ± 3
	4 hr	6	7 ± 5	75 ± 8	11 ± 5	5 ± 1
	24 hr	2	72 ± 2	13 ± 1	4 ± 2	5 ± 0
IA4 (100 μg/g)	4 hr	3	87 ± 4	7 ± 3	2 ± 2	0
	24 hr	2	80 ± 4	11 ± 3	2 ± 2	1 ± 1

[a] Fraction number.

after its administration (Table 3). In one experiment, IA4-*N*-oxide (100 μg/g) did not induce slow sedimentation of liver DNA in either neutral or alkaline sucrose gradients at 1, 4, or 24 hr. Niridazole (200 μg/g of body weight), another antischistosomal drug, structurally different from hycanthone derivatives, also did not cause any change in liver DNA as measured by sedimentation in neutral or alkaline sucrose gradients at 1, 4, or 24 hr.

Since hycanthone has been reported to intercalate into DNA (31–33), it was of interest to determine whether any effect of this compound could be observed on the melting profile of the hepatic DNA of rats treated with hycanthone methanesulfonate. The T_m (temperature at which the hyperchromic effect is 50% of its maximal value) of the DNA isolated from the livers of rats treated with hycanthone methanesulfonate (150 μg/g of body weight) for 4 hr showed no change either in 0.15 M NaCl–0.015 M sodium citrate, pH 7.5, or at lower ionic strength (0.0015 M NaCl–0.00015 M sodium citrate, pH 7.5), perhaps because the intercalated drug was washed out during the isolation of DNA. However, the T_m of calf thymus DNA (20 μg/ml) incubated *in vitro* with hycanthone methanesulfonate (10 μg/ml) in the low ionic strength medium showed a shift from 66° to 75°, and a similar pattern was observed when rat liver DNA was used in place of calf thymus DNA. This increase in T_m could be observed only when the thermal denaturation of calf thymus or rat liver DNA (20 μg/ml) was carried out in the low ionic strength buffer but not in 0.15 M NaCl–0.015 M sodium citrate. IA4 (10 μg/ml) also increased the T_m of rat liver DNA (20 μg/ml) from 64° to 82° when incubated in the low ionic strength buffer *in vitro*. Similarly, IA4-*N*-oxide (10 μg/ml) in the low ionic strength buffer increased the T_m of rat liver DNA from 62° to 68°. Niridazole, in contrast, had no effect on the T_m of rat liver DNA.

DISCUSSION

It is evident from this study that the antischistosomal drug hycanthone causes damage in rat liver DNA *in vivo*, which can be monitored as slow sedimentation of DNA in alkaline and neutral sucrose gradients. Two analogues of hycanthone, IA4 and IA4-*N*-oxide, which are less hepatotoxic, teratogenic, and mutagenic than hycanthone (22–27), and another antischistosomal drug, niridazole (34), which is structurally different from hycanthone, failed to induce slow sedimentation of liver DNA in neutral sucrose gradients. Our studies to date indicate that induction of hepatic DNA damage, resulting in the slow sedimentation of liver DNA in neutral sucrose gradients, is a property of a very few chemicals, and these so far have been liver carcinogens (13–15, 17, 19, 20): *N*-hydroxy-2-acetylaminofluorene, *N*-acetoxy-2-acetylaminofluorene, *N*-nitrosomorpholine, *N*-nitrosodihydrouracil, and 3-hydroxyxanthine. The demonstrated hepatocarcinogenicity of hycanthone methanesulfonate in mice with schistosomiasis (8) indicates that this drug may also fall into this category.

The mechanism by which hycanthone induces slow sedimentation of liver DNA in alkaline and neutral sucrose gradients is not clear at present. During metabolism, hycanthone loses its ethyl groups from the terminal nitrogen (6), and it has not yet been established whether these ethyl groups are utilized to ethylate purines and/or the phosphodiester backbone of DNA. This is of interest because several alkylating agents, both methylating and ethylating, cause strand breaks in liver DNA *in vivo* (11–15, 18). Hulbert and Miller (35) have suggested that hycanthone may be metabolized to yield a carbonium ion which is a potential intermediate in alkylation. Thus hycanthone may alkylate liver DNA purines and/or the phosphodiester backbone to yield phosphotriester. Removal of the alkylated purines *in vivo*, either enzymatically by *N*-glycosidases (36) or nonenzymatically, results in apurinic sites (37). Both apurinic sites and phosphotriesters in DNA are alkali-sensitive (37–39) and could be responsible for the observed slow sedimentation of liver DNA in alkaline sucrose gradients.

In a recent study, niridazole has been shown to induce DNA strand breaks in

Salmonella typhimurium strain TA1975 (UVR^+) (40). The absence of such induction of DNA strand breaks in rat liver DNA *in vivo* could be due to insufficient interaction of the drug with DNA, such as 1 molecule/1 $\times 10^7$ nucleotides, giving rise to a minimal number of breaks. A molecular weight of DNA of 3×10^9 resulting from such breaks is rather difficult to detect under the conditions of sedimentation in the gradients, because such DNA will sediment in the 2.3 M sucrose layer (13). The inability of niridazole to induce strand breaks in liver DNA may also be due either to an absence of its metabolic activation (if required) or to detoxification of the drug in the liver.

In conclusion, neither the ability of chemicals to induce slow sedimentation of DNA in neutral and alkaline sucrose gradients nor their repair can as yet be related unequivocally to their carcinogenic potential *in vivo*. Nevertheless, the antischistosomal drug hycanthone resembles several hepatocarcinogens in its effects *in vivo* on liver DNA.

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