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STUDIES ON DITHRANOL AND DITHRANOL-LIKE COMPOUNDS

II. *Mutagenicity*

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Abstract. Eleven anthracene and anthraquinone derivatives have been tested for their ability to induce respiration-deficient (RD-) mutants in yeast. Dithranol (1,8,9,-trihydroxy-anthracene) was shown to be highly effective in very low concentrations. 1,9-dihydroxy-anthracene and 1,8,9-trihydroxy-3-methyl-anthracene also have the same property, but to a lesser extent. After prolonged use, 1,8-dihydroxy-anthraquinone and 1,8-diacetoxy-anthraquinone also induced a low but significant increase in the proportion of RD-mutants. Compounds which induce RD-mutants are all strong DNA-binders in vitro. Anthraquinone derivatives do not seem to be as effective in RD-induction although many are strong DNA-binders in vitro. The frequency of chromosomal mutations was not increased by treatments with dithranol on yeast or *Ophiostoma*. The mechanism for the induction of RD-mutation with the compounds is discussed and a hypothesis is presented to explain the antipsoriatic effect of dithranol.

As has been shown earlier (11, 13, 15) dithranol and dithranol-like compounds interact with native DNA. It has been assumed and considered likely that this interaction is important for the antipsoriatic effect of dithranol (11, 12).

An in vitro effect of dithranol on a more complicated biological system where DNA is involved has been shown by Lukács (6). An interaction between a chemical and DNA often gives rise to mutations and in an earlier paper we showed that dithranol readily induces respiratory-deficient mutants in yeast (3). In the present investigation we have studied whether there is a correlation between DNA-binding and mutagenicity for dithranol-like compounds. Riva (9) found no correlation between DNA-binding and the mutagenic effects of acridines.

The use of mutagenic substances in the treatment of patients gives rise to some concern. Though dithranol has been used as an antipsoriatic agent for more than 50 years, no side-effects that may be directly attributed to its mutagenic properties have been reported. In a study of squamous cell skin cancer in Sweden, Swanbeck & Hillström (14) found no significant overrepresentation of skin cancer in psoriatic patients despite the lengthy use of dithranol in Sweden.

Mitochondria from practically all kinds of living material have been found to contain DNA. This DNA has been shown to provide information for enzymes in the respiratory chain. Mitochondria have an autonomous nature: a cell cannot regenerate lost mitochondria. In experiments with yeast and *Neurospora* cells it has been shown that respiration-deficient mutants (RD-mutants) lack certain active enzymes in the terminal respiratory chain. Thus, such mutants can grow only by fermentation.

The mutagenic effect of dithranol shown earlier (3) may only involve mitochondria. Thus the very high frequencies of RD-mutants obtained strongly indicate that the mutants were of non-chromosomal origin. In the present study we have tried to obtain nuclear mutations by dithranol treatment, the most effective compound for the induction of RD-mutants.

MATERIAL AND METHODS

RD-mutation experiments in yeast

The frequency of RD-mutants was studied in a diploid prototrophic strain of *Saccharomyces cerevisiae* selected on minimal medium after crossing two auxotrophic mutants. Cells from an overnight culture were used to inocu-

Table I. *Per cent RD-mutants in Saccharomyces cerevisiae induced by treatment with anthracene- and anthraquinone derivatives*

Treatment at 30°C for 16–18 h. Degree of significance (using *t*-test) indicated with * $p = 0.05$; ** $p = 0.01$; *** $p = 0.001$
Numbers within parentheses indicate the number of the substance in Tables I and II of ref. (15)

Substance tested		Concentration in per cent of a saturated solution in 96% ethanol								
		0.5	0.2	0.1	0.04	0.02	0.008	0.004	0.0008	Control
9-hydroxy-	(*a*)			3.5		3.3		3.2	4.0	4.4
anthracene (1)	(*b*)	7.05	4.62							6.08
1.9 dihydroxy-	(*a*)			12.10***		6.22		6.28	9.64*	4.99
anthracene (2)	(*b*)	58.7***	30.0***							5.6
1,8,9-trihydroxy-	(*a*)			15.17***		5.49*		4.85	4.11	3.19
3-methylanthracene (5)	(*b*)	67.7***	17.1***							5.6
1-hydroxy-antharaquinone (7)		4.46	2.47		1.89		1.56			2.32
1.4-dihydroxy-anthraquinone (9)		3.24	1.75		2.17		1.70			2.75
1.5-dihydroxy-anthraquinone (10)		4.72*	2.62		1.93		3.01			2.40
2.6-dihydroxy-	(*a*)			2.2		3.4		3.7	4.1	3.0
anthraquinone (12)	(*b*)	6.78	5.95							6.08
1-methoxy-anthraquinone (17)		2.91	1.75		1.68		1.88			3.10
1-amino-	(*a*)			2.86		2.18		2.10	1.69	2.00
anthraquinone (18)	(*b*)	6.18	5.53							6.08
1.8-diacethoxy-	(*a*)			4.09*		2.64		2.08	3.66	2.35
anthraquinone (20)	(*b*)	4.72	2.25		1.79					3.61

late 20 ml of complete medium.[1] The compound to be tested was dissolved in 96% ethanol, diluted in ethanol and 0.9% NaCl and added to the yeast culture to give the final concentrations indicated in the tables. A control culture started simultaneously contained ethanol at the same concentration as the culture with the highest concentration of the compound to be tested (max. 5%). The cultures were incubated in darkness at 30°C on a shaker for 16–18 hours, unless otherwise indicated. The treatment was stopped by washing the cells twice in salt solution. After dilution the cells were plated on a medium containing sodium tellurite (8) and incubated for 3 days at 30°C. Colonies of respiration-competent cells are black while RD-mutants form small white colonies. Eight replicates of plates were carried in each series.

Deviations from the results obtained in the control series were statistically tested using the *t*-test.

[1] Complete medium for *Saccharomyces*: (per litre) glucose 40 g, peptone 3 g, yeast extract 5 g, hydrol. casein 5 g, vitamins as in minimal medium (below). pH 5.8.

[2] Minimal medium for *Saccharomyces*: (per litre) glucose 40 g, $MgSO_4 \times 7H_2O$ 0.5 g, NH_4Cl 2.0 g, $CaCl_2$ 0.5 g, KH_2PO_4 1.5 g, $FeSO_4 \times 7\ H_2O$ 0.3 mg, $KMnO_4$ 0.03 mg, $ZnSO_4 \times 7\ H_2O$ 0.4 mg, $CoNO_3 \times 6\ H_2O$ 0.05 mg, KJ 0.1 mg, inositol 1 mg, thiamin 0.2 mg, niacin 0.2 mg, riboflavin 0.2 mg, Ca-panthotenate 0.2 mg, PABA 0.1 mg, pyridoxin 0.2 mg, biotin 5 μg. pH 5.8.

Back-mutation experiments in yeast

When back-mutations to prototrophy were studied in an adenine-requiring strain, ad-2.7, of *Saccharomyces cerevisiae*, treatment was similar to that in the experiments for the induction of RD-mutants. After treatment, the cells were washed and 0.1 ml (about 2×10^7 cells) of the cell suspension was plated on each of 10 plates of minimal medium[2] to select for reversions to prototrophy. A viable count was made on complete medium. In some experiments we also treated cell suspensions which had a density of about 10^7 cells/ml from the beginning of the treatment. During incubation (treatment) the cells in these suspensions should not be able to divide more then two or three times, whereas in treatments previously described, a small inoculum (0.25 ml of an overnight culture) was added to 20 ml of medium and several cell generations passed during the treatment.

Back-mutation experiments in Ophiostoma

Induction of back-mutations was also tested in the ascomycete *Ophiostoma multiannulatum* using a methionineless strain, no. 2511. Cells were grown in minimal medium (2) + methionine (50 mg/l) to the late logarithmic phase, filtered, washed and suspended in salt solution. After treatment the cells were plated on minimal medium to select for revertants to protrophy. Viable counts were made on minimal medium + methionine.

Forward-mutation experiments in Ophiostoma

In forward-mutation tests with *Ophiostoma multiannulatum* a prototrophic strain, no. 14, was used. Cells were grown in minimal medium to the late logarithmic phase. After filtration through cotton wool the cells were washed and resuspended in 0.9% NaCl to a density of 4×10^7 cells/ml. The treatment was made in flasks placed on a shaker at 30°C in darkness and lasted for 3 hours. After repeated washings in salt solution and proper dilution the cells were plated on complete medium[3] and incubated at 25°C for 5 days. The colonies were transferred to minimal medium and tested for induced auxotrophy.

RESULTS

In order to compare binding to DNA and mutagenicity of dithranol-like compounds the induction of respiratory-deficient (RD) mutants in *Saccharomyces cerevisiae* was studied. With the help of our previously reported data on complex formation with DNA we chose certain substances that form such complexes and some that do not. As shown in Table I a high frequency of RD-mutants was induced only by 1,9-dihydroxy-anthracene and 1,8,9-trihydroxy-3-methyl-anthracene.

In the series of experiments reported in Table I no anthraquinone derivative induced a high frequency of RD-mutants. Chrysazine (1,8-dihydroxy-anthraquinone) very readily forms a complex with DNA (15), and after 1 day in a water solution 1,8-diacetoxy-anthraquinone hydrolyses to chrysazine which is then bound to DNA. We therefore made a series of experiments with these two substances for several days and at different concentrations (Tables II and III). An induction of RD-mutants occurred but to a much lesser degree than for dithranol. In these experiments also, a rather high frequency of sectored colonies was registered which indicates an induced instability of the mitochondrial material in the treated cells.

In two series of experiments the concentration in mole/litre of dithranol necessary for the induction of RD-mutants was investigated (Table IV). About 2.5×10^{-7} M/l of dithranol is the lowest concentration that eliminates all mitochondria in 50% of the cells during an 18 hour treatment.

[3] Complete medium for *Ophiostoma:* 1 litre minimal medium + malt extract 25 g, yeast extract 2.5 g, NZ-case 2.5 g. pH 5.6.

Table II. *Induction of RD-mutants in Saccharomyces cerevisiae by treatment with 1,8-dihydroxy-anthraquinone*

The cells were incubated at 30°C in complete medium containing the conc. indicated (in per cent of a saturated ethanol solution). Degree of significance indicated as in Table I

Duration of treatment (days)	Per cent RD-mutants induced by the concentrations			
	Control	0.04%	0.2%	0.5%
1	3.41	2.50	3.44	4.30
2	2.82	2.03	2.38	5.79*
3	3.59	1.83	2.16	4.99
4	3.75	1.86	2.63	4.50
	Per cent sectored colonies			
1	1.29	1.07	1.98	3.44*
2	2.03	0.81	1.49	5.27**
3	1.63	0.92	1.65	3.55*
4	1.27	1.52	1.55	2.51*

Table III. *Induction of RD-mutants in Sacharomyces cerevisiae by treatments with 1,8-diacetoxy-anthraquinone*

The cells were incubated at 30°C in complete medium containing the conc. (in per cent of a saturated ethanol solution) indicated. Degree of significance indicated as in Table I

Duration of treatment (days)	Per cent RD-mutants induced by 1,8-diacetoxy-anthraquinone of the concentrations			
	Control	0.04%	0.2%	0.5%
1	3.42	1.86	3.28	3.73
2	2.19	1.91	2.41	4.86**
3	3.82	2.24	2.87	7.56**
4	3.18	2.14	3.15	6.86**
	Per cent sectored colonies after 3 and 4 days' treatment			
3	0.79	0.76	1.28	4.70***
4	0.84	0.50	1.30	3.80***

Table IV. *Per cent RD-mutants induced in yeast by 1,8,9-trihydroxy-anthracene (dithranol) during an 18 hour treatment*

Expt. no.	Conc. dithranol moles/l	Per cent RD-mutants
1	3.3×10^{-6}	92.0
	3.3×10^{-7}	77.5
	3.3×10^{-8}	7.7
	0	3.2
	1.65×10^{-7}	13.2
	6.6×10^{-8}	8.5
	3.3×10^{-8}	6.4
	0	5.9

Table V. *The effect of 1,8,9-trihydroxy-anthracene (dithranol) on the frequency of reversions to ad^+ in an ad^- strain of Saccharomyces cerevisiae*

A. Treatment of a dense suspension (ca 2×10^7 cells/ml at the start of the treatment)
B. Treatment of a growing culture

Type of treatment	Exp. no.	Conc. of dithranol in % of a sat. soln	Duration of treat- ment (hours)	Number of cells inves- tigated for revertants	Absolute number of revertants
A	1	0.2	18	1.3×10^8	2
		0.04		1.2×10^8	3
		control		1.5×10^8	1
	2	0.2	18	1.1×10^8	2
		0.04		1.1×10^8	3
		control		1.1×10^8	2
B	1	0.2	18	1.7×10^8	13
		0.04		1.3×10^8	0
		control		1.4×10^8	0
	2	0.2	18	1.3×10^8	2
		0.04		1.4×10^8	0
		control		1.5×10^8	1
	3	0.2	18	1.1×10^9	14
		0.04		2.1×10^9	3
		control		1.0×10^9	10
	4	0.2	70	9.0×10^7	2
		0.04		7.5×10^7	0
		control		8.0×10^7	3

In three series of experiments attempts were made to induce chromosomal mutations with dithranol. In no case was any mutagenic effect on nuclear genes found (Tables V, VI and VII).

Table VI. *The effect of 1,8,9-trihydroxy-anthracene (dithranol) on the frequency of reversions to $meth^+$ in a $meth^-$ strain of Ophiostoma multiannulatum*

Conc. of dithranol in % of a sat. soln	Duration of treatment (hours)	Number of cells inves- tigated for revertants	Per cent survivors	Absolute number of revertants
0.0125	3	2.5×10^7	61	3
0.0025	3	2.3×10^7	70	3
0.0006	3	2.3×10^7	69	1
0	3	3.3×10^7	100	1

Table VII. *The effect of dithranol on the frequency of forward mutations in a wild-type strain of Ophiostoma multiannulatum*

Conc. in % of a saturated solution	Duration of treatment (hours)	Per cent survivors	Number of isolates tested	Number of mutants induced
0.1	3	5.8	1.600	0

DISCUSSION

Anthracene derivatives which induced RD-mutants in yeast also showed strong binding to DNA (15). None of the anthraquinone derivatives tested seems to induce RD-mutants so readily as do some anthracene derivatives. Thus, one of the best DNA binders, 1,8-dihydroxy-anthraquinone (chrysazin), was shown to induce RD-mutants, although to a very low extent. From the data presented here and in our earlier paper on binding to DNA it may be concluded that for anthracene and anthraquinone derivatives a necessary condition for RD-mutagenic effect on yeast is binding to DNA in vitro but that not all derivatives which bind to DNA can be shown to induce RD-mutants. The correlation between the antipsoriatic effect of different derivatives as shown by Krebs & Schaltegger (4, 5) and our mutagenic data seems, however, to be very good.

In spite of repeated experiments on different systems we have not been able to induce chromosomal mutants with dithranol. In the microorganisms studied, only the mitochondrial genes seem to have been affected. It has been shown (10, 16) that acridines bind to a greater extent to mitochondrial DNA than to nuclear DNA. This may

also be the case with dithranol which was highly effective in induction of RD-mutants but non-effective in induction of nuclear mutations. It could follow from such a selective binding that the replication of mitochondrial DNA is prevented while the nuclear DNA is not affected in a similar way. Thus in the case of yeast, which is capable of fermentation, nuclear division could proceed, but not the replication of mitochondria. Such a cell would give rise to a RD-colony. Meyer & Simpson (7) have found that acriflavine, a dye which is extremely potent in induction of RD-mutants in yeast, specifically inhibits mitochondrial DNA polymerase from rat liver while nuclear DNA polymerase is much less sensitive to the inhibition of acriflavine. If dithranol has a similar effect, which we have not tested, this could be an interpretation of its potency in induction of RD-mutants and the absence of effect on nuclear DNA.

It is tempting to believe that dithranol has the same effect on psoriatic epidermal cells as it has on yeast. Its primary point of attack should thus be the DNA of the epidermal mitochondria. This hypothesis, however, remains to be proved.

Dithranol does not seem to be a carcinogen in man (14), though it functions as a co-carcinogen in mice (1). We do not know in which genes a mutation would lead to cancerous growth. A few mitochondrial mutations, or a retarded replication of mitochondrial DNA, might not be dangerous to an epidermal cell, but may slow down the somewhat rapid metabolism and growth of a psoriatic cell. Today this seems to us the most plausible interpretation of the antipsoristic effect of dithranol.

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