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BINDING OF 4'-AMINOMETHYL 4,5',8-TRIMETHYL PSORALEN TO DNA, RNA AND PROTEIN IN HeLa CELLS AND *DROSOPHILA* CELLS

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Summary

In *Drosophila* cells and HeLa cells treated with 4'-aminomethyl trioxsalen and ultraviolet light, this compound binds covalently to DNA and RNA. The maximum number of molecules bound to 10^3 base pairs in DNA is 60 and in RNA it is 20. In nuclei treated likewise the number of molecules bound to 10^3 base pairs in DNA can be as high as 376. When cells are irradiated in the frozen state the number of 4'-aminomethyl trioxsalen molecules bound per 10^3 base pairs in DNA is about 40 and in RNA about 20.

DNA molecules from cells or nuclei treated with 4'-aminomethyl trioxsalen and ultraviolet light are highly crosslinked and appear as loops interspersed by double stranded regions when analyzed in the electron microscope under denaturing conditions. The loop sizes are heterogeneous and the fraction of double stranded regions increases to almost complete double-strandedness at high degrees of reaction.

No sècondary structures could be found in ribosomal RNA from *Drosophila* cells or HeLa cells after treatment with 4'-aminomethyl trioxsalen and ultraviolet light.

In cells treated with 4'-aminomethyl trioxsalen and ultraviolet light the RNAase activity is increased considerably suggesting a release of lysosomal enzymes.

4'-aminomethyl trioxsalen and its photodecomposition products bind strongly to cellular proteins.

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Introduction

Different compounds of the furocoumarin family known as the psoralens have clinical applications. The photochemistry and photobiology of furocoumarins are reviewed in Refs. 1—4. 8-Methoxypsoralen or trioxsalen and ultraviolet light have been used in the treatment of infections with mucosis fungosides [5], vitelligo [6,7] and psoriasis [8—10]. The inhibition of cell proliferation in the psoriatic skin is believed to be due to the crosslinkage of the two DNA strands by photo-addition of psoralen. In fact the formation of DNA crosslinks have been demonstrated in different cells [11—13] and in skin cells as well [14,15]. Psoralens are planar molecules which can intercalate between base pairs in DNA. When irradiated with ultraviolet light (320—380 nm) either one or two cyclobutane bridges may be formed between psoralen and pyrimidines in DNA [11,16]. Diadducts will crosslink the two strands in DNA and such structures can be seen as looped molecules when DNA is examined in the electron microscope under denaturing conditions [12,13,17—19].

Different psoralens have been shown to photoreact with tRNA and rRNA [20—24] and trioxsalen has been shown to photoreact with RNA in skin cells [25]. The extent of reaction with RNA was low in all cases and much lower than the reaction with DNA. This drawback was circumvented recently by the synthesis of new psoralen derivatives [26]. Two of these compounds, 4′-aminomethyl 4,5′,8-trimethyl psoralen (4′-aminomethyl trioxsalen) and 4′-hydroxymethyl 4,5′,8-trimethyl psoralen bind much stronger to isolated DNA and RNA [26,27]. DNA and RNA viruses has been inactivated by treatment with these two compounds plus ultraviolet light [28] and the DNA-RNA hybrid helix can be crosslinked by these psoralen derivatives [29]. Trioxsalen has previously been used as a probe for chromatin structure [12,13,17,30,31]. The solubility of this compound is low, however, and this necessitates several additions of the compound followed by irradiation. Since sliding of histones on chromatin DNA has been demonstrated under certain conditions [32,33] it is difficult to rule out that thistones do not slide during the repeated irradiation. The high solubility of 4′-aminomethyl trioxsalen [26] suggested this compound to be a good candidate as a probe for nucleic acid structure within the cells. The present paper describes the binding of 4′-aminomethyl trioxsalen to DNA, RNA and proteins in HeLa cells and *Drosophila* cells.

Materials and Methods

4′-Aminomethyl 4,5′,8-trimethyl psoralen (4′-aminomethyl trioxsalen) and the tritiated compound were synthesized by Isaacs et al. [26].

Radioactivity was measured in a mixture of 1 l Triton X-100/2 l toluene/12 g Omnifluor (New England Nuclear) made 10% in H_2O.

Ribonuclease (5 × crystallized, A grade) was obtained from Calbiochem. Pronase (B grade, Calbiochem) was incubated for 1 h at 37°C in sodium citrate buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) before use.

Drosophila melanogaster cells (Schneider line 2) [34] were grown in monolayer cultures in Echalier's medium [35] containing 15% fetal calf serum

(heated 30 min at 60°C). The cells were harvested with a rubber policeman, centrifuged at 500 × *g* for 5 min. The cell pellet was suspended in a small volume of medium and incubated at 25°C with stirring.

HeLa cells were grown in suspension cultured in Minimal Essential Medium Joklick modified spinner medium and kindly provided by Jane Smith. The cells were collected by centrifugation at 500 × *g* for 5 min, resuspended in small volume of medium and incubated at 37°C with stirring.

Irradiation. After incubation with 4'-aminomethyl trioxsalen the cell suspension was spread on a aluminum or glass petri dish in a layer of about 1 mm thickness. The petri dish which was pre-cooled to 0°C was then transferred to a copper block kept in a mixture of methanol and dry ice. The cell suspension was frozen within 15 s and irradiation was performed while the cells were kept at −78°C. Four General Electric BLB blacklights giving ultraviolet light in the range from 320—380 nm were used for irradiation. The intensity was 3.2 $mW \cdot cm^{-2}$ and about 2/3 of the light passed through the frozen cell suspension.

Cell suspensions were irradiated with ultraviolet light (340—380 nm) at 100 $mW \cdot cm^{-2}$ while stirred at 0—5°C. This high intensity irradiation device contained two 400-W General Electric mercury-vapor lamps and a filter of cobaltous nitrate solution (40%, w/w) [26].

Isolation of RNA. Aqueous solutions used for RNA experiments were routinely treated with diethyl pyrocarbonate. RNA was extracted at 0°C with phenol and reticulocyte standard buffer (0.01 M NaCl/1.5 mM $MgCl_2$/0.01 M Tris-HCl, pH 7.4) containing diethyl pyrocarbonate (10 µl per $4 \cdot 10^8$ cells) or RNA was extracted at 55°C with phenol and 0.5% sodium dodecyl sulphate in 0.05 M acetate buffer, pH 5.1 [36].

Purified RNA (10—12 A_{260} units) was layered on top of a 5—20% sucrose gradient (w/v) containing 0.05 M Tris-HCl, pH 7.4, 1 mM EDTA. The gradients were centrifuged for 18 h (HeLa RNA) or 19 h (*Drosophila* RNA) in a Spinco SW 25 rotor at 22 000 rev./min (average 55 000 × *g*).

Isolation of DNA. Irradiated *Drosophila* cells were thawed and centrifuged for 5 min at 900 × *g* and the cells then resuspended in 0.5% Nonidet P-40 in sodium citrate buffer (0°C). Treatment for 2 × 15 s in a Vortex mixer at maximum speed leaves less than 1% cells unbroken. The nuclei were collected by centrifugation at 1000 × *g* for 5 min and lyzed in 1.5% sarkosyl in sodium citrate buffer containing 5 mM EDTA. After 2 × 15 s treatment on a Vortex mixer, pronase was added to the suspension (final concentration 1 $mg \cdot ml^{-1}$) and incubated 5—7 h at 50°C. The nuclear digest was made 1.0 M in NaCl and extracted once with 1 vol. of chloroform-isoamyl alcohol (24 : 1). The aqueous phase was dialyzed overnight at 0°C against sodium citrate buffer. RNAase was added to the dialyzed solution (final concentration 150 $\mu g \cdot ml^{-1}$), incubated 3 h at 37°C, pronase added (final concentration 1 $mg \cdot ml^{-1}$) and incubation continued for 4 h. The solution was extracted once with chloroform-isoamyl alcohol and the aqueous phase dialyzed overnight at 0°C against 0.01 M sodium phosphate buffer, 1 mM EDTA, pH 6.8. This procedure is a modification of that reported by Wiesehahn et al. [13].

DNA was in some cases obtained from the interphase remaining after phenol extraction of cells. The interphase was purified by further phenol extraction, washed with alcohol and treated as above with pronase and RNAase.

Electron microscopy. The purified DNA samples were denatured in 2.5% formaldehyde (w/v), 10 mM sodium phosphate buffer (pH 6.8), 1 mM EDTA, 70% formamide (v/v) at 37°C for 30 min and then spread for electron microscopy using the method of Davis et al. [37]. Purified RNA was denatured in 2.5% formaldehyde (w/v), 5 mM Tris-HCl pH 8.5, 0.1 mM EDTA, 87% formamide (v/v) at 50°C for 10 min. Spreading was done as for DNA except that the hyperphase consisted of 10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 49% formamide (v/v) and the hypophase consisted of 17% formamide (v/v) in the same buffer. RNA samples were shadowed with tungsten. Single-stranded fd DNA shown to contain 6400 nucleotides [38] was used as internal length marker. Length measurements were performed on enlarged projections of 35 mm negatives using an electronic planimeter (Numonics Corp,). Microscopy was performed on a Philips 201 electron microscope.

Binding of ^{3}H-labeled 4′-aminomethyl trioxsalen to protein. Drosophila cells were taken through the modified Smith-Tannhauser washing procedure using 2 ml of each solution per $2.5 \cdot 10^8$ cells. [39]. The final precipitate of proteins was dissolved in 0.3 M KOH, neutralized and the radioactivity measured.

Results

Photosensitizers like anthracene and other polybenzoid hydrocarbons are concentrated in the lysosomes and after irradiation with ultraviolet light give rise to an increased release of degradative enzymes from the lysosomes [40,41]. Evidence for an increased release of proteolytic enzymes from lysosomes by 8-methoxypsoralen and ultraviolet light has been presented [42]. The possibility that 4′-aminomethyl trioxsalen could cause a release of nucleases from the lysosomes is of obvious importance in studies of DNA and RNA structures in situ and the extent of degradation of RNA during the treatment was therefore investigated.

HeLa cells were incubated with 4′-aminomethyl trioxsalen and then irradiated in a high intensity irradiations device ($100\ mW \cdot cm^{-2}$, 340—380 nm) for 10 min at 0—5°C. The cells were lysed with buffer containing Nonidet P-40 and the homogenate separated into a nuclear and a cytoplasmic fraction by centrifugation. RNA was extracted from the cytoplasmic fraction with phenol and centrifuged on sucrose gradients. Irradiation alone has some deteriorating effect on the RNA profile as seen from the decreased 28 S/18 S ratio (Fig. 1B). RNA from 4′-aminomethyl trioxsalen and ultraviolet light treated cells shows a pronounced degradation of 28 S RNA and this peak is now smaller than the 18 S peak (Fig. 1C).

The rRNA in *Drosophila* cells treated as above is even more seriously degraded. In cells treated with 4′-aminomethyl trioxsalen and ultraviolet light all the RNA extracted from the cytoplasm is smaller than tRNA when centrifuged in a sucrose gradient (results not shown).

The effect of the RNAases can be minimized if handling of the cells is avoided after 4′-aminomethyl trioxsalen and ultraviolet treatment. RNA was therefore extracted from whole cells immediately after the treatment. The RNAase is, however, still active enough to give some degradation if RNA is extracted with reticulocyte standard buffer and phenol at 0°C (Fig. 2C). If

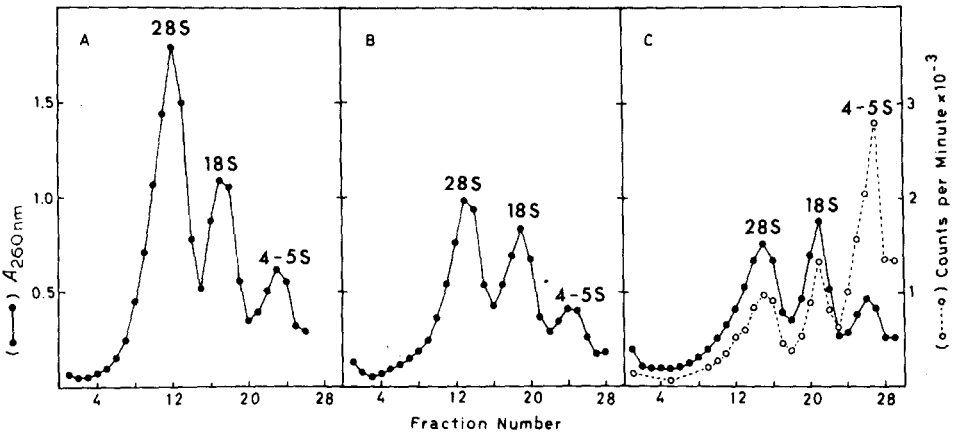

Fig. 1. Effect of 4′-aminomethyl trioxsalen and ultraviolet light on the degradation of RNA in HeLa cell cytoplasm. HeLa cells (10^7 cells · ml^{-1}) were incubated for 20 min at 37°C with ^{3}H-labeled 4′-aminomethyl trioxsalen (143 $\mu g \cdot ml^{-1}$ spec. act. $3.453 \cdot 10^{12}$ cpm · mol^{-1}). The suspension was then chilled in ice and irradiated for 10 min at 0°C with ultraviolet light (100 mW · cm^{-2}, 340—380 nm). The cells were suspended in 0.5 × sodium citrate buffer, 1% nonidet-P-40 with diethyl pyrocarbonate, homogenized in a Dounce homogenizer and then centrifuged for 10 min at 1000 × *g*. The cytoplasmic RNA was extracted from the supernatant with phenol, purified and centrifuged in a 5—20% sucrose gradient. A, control cells; B, cells treated with ultraviolet light; C, cells treated with ^{3}H-labeled 4′-aminomethyl trioxsalen and ultraviolet light.

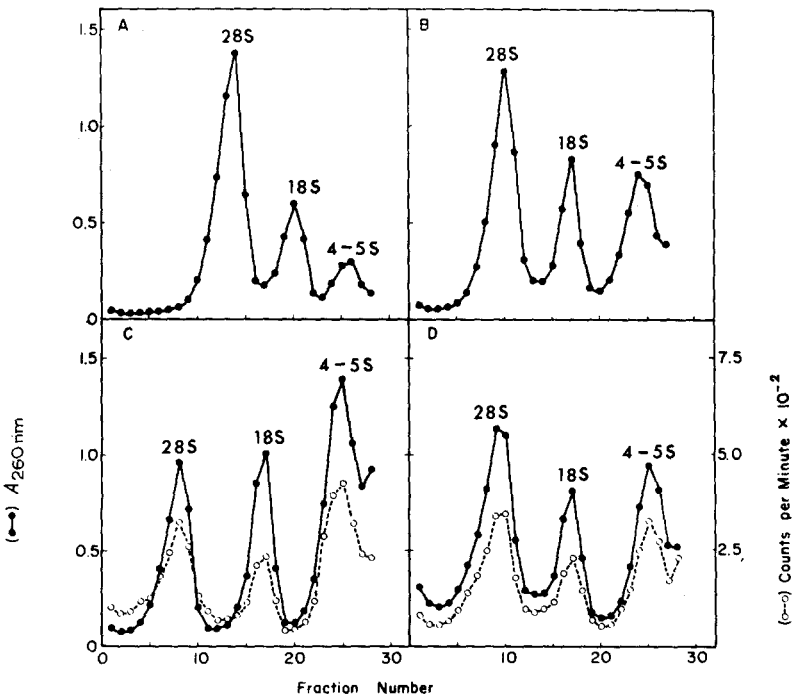

Fig. 2. Effect of 4′-aminomethyl trioxsalen and ultraviolet light on the degradation of RNA in HeLa cells. HeLa cells were incubated, irradiated and centrifuged on sucrose gradients as described in Fig. 1, but whole cells were extracted with reticulocyte standard buffer and phenol at 0°C (A, B and C) or with 0.5% sodium dodecyl sulphate in acetate buffer pH 5.2 at 55°C (D). A, untreated control cells; B, ultraviolet light treated cells; C, ^{3}H-labeled 4′-aminomethyl trioxsalen and ultraviolet light treated cells; D, ^{3}H-labeled 4′-aminomethyl trioxsalen and ultraviolet light treated cells.

RNA is extracted with sodium dodecyl sulphate in sodium acetate buffer pH 5.2 at 55°C the RNA profile on a sucrose gradient (Fig. 2D) looks very much like the RNA obtained from cells irradiated without the psoralen (Fig. 2B). Both profiles show a little more material in the 4—5 S peak than RNA from untreated controls (Fig. 2A). The 4—5 S peak is larger when RNA is extracted at 55°C because the 5.8 S ribosomal RNA is released from the 28 S RNA (Fig. 2D).

The degradation of RNA in *Drosophila* cells could not be prevented completely by these phenol extractions, when cells were irradiated in suspensions at 0—5°C. Some degradation seems to occur during the 5 min of irradiation. Degradation was, however, avoided when *Drosophila* cells were irradiated in the frozen state and then extracted with reticulocyte standard buffer and phenol at 0°C (Fig. 4A).

In order to study the structure of RNA and DNA within the cell it was necessary to find the optimal conditions for binding of 4′-aminomethyl trioxsalen to the nucleic acids during irradiation in the frozen state. *Drosophila* cells ($2 \cdot 10^8$ cells per ml) were incubated in suspension with 4′-aminomethyl trioxsalen (spec. act. $3.45 \cdot 10^{12}$ cpm $\cdot$ mol^{-1}) for 30 min, frozen to minus 78°C within 15 s and then irradiated in the frozen state with a low intensity black light irradiation device (320—280 nm, 3.2 mW $\cdot$ cm^{-2}). Cells were irradiated for 4, 8 and 17 h and the number of 4′-aminomethyl trioxsalen molecules bound to 10^3 base pairs of DNA was found to be 17.8, 23.6 and 26.4, respectively. The number of molecules bound to 10^3 base pairs of RNA was 10.4, 12.2 and

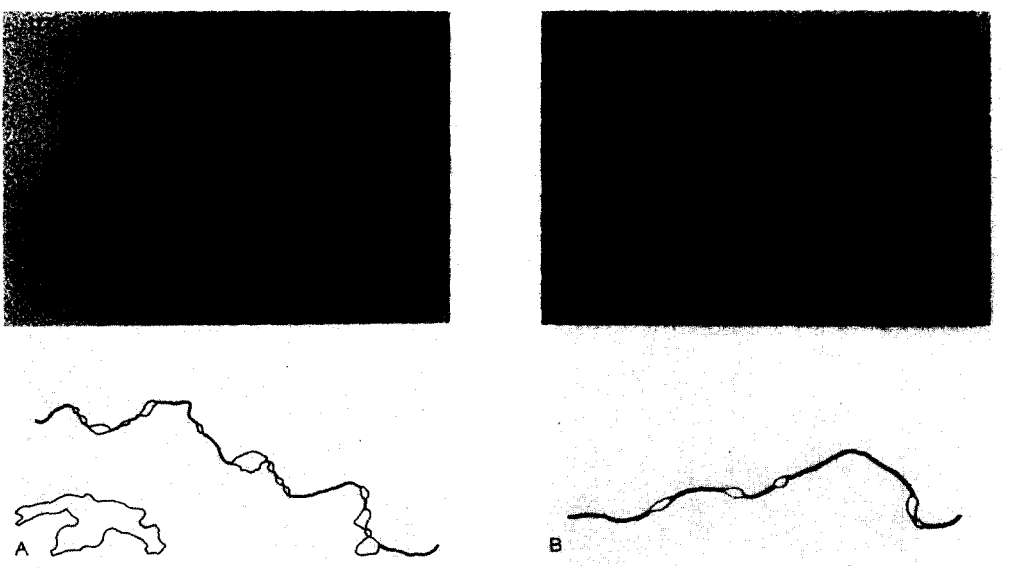

Fig. 3. Electron micrographs of denatured DNA from *Drosophila* cells and nuclei treated with 4′-aminomethyl trioxsalen and ultraviolet light. Total magnification 112 500×. Circular fd DNA containing 6400 nucleotides [38] was used as internal marker. A, DNA having 34 molecules 4′-aminomethyl trioxsalen bound per 10^3 base pairs. Cells treated as described in Table I, Expt. 2; B, DNA having 376 molecules 4′-aminomethyl trioxsalen bound per 10^3 base pairs. This molecule contains more loops than the average. Nuclei treated as described in Table I, Expt. 4.

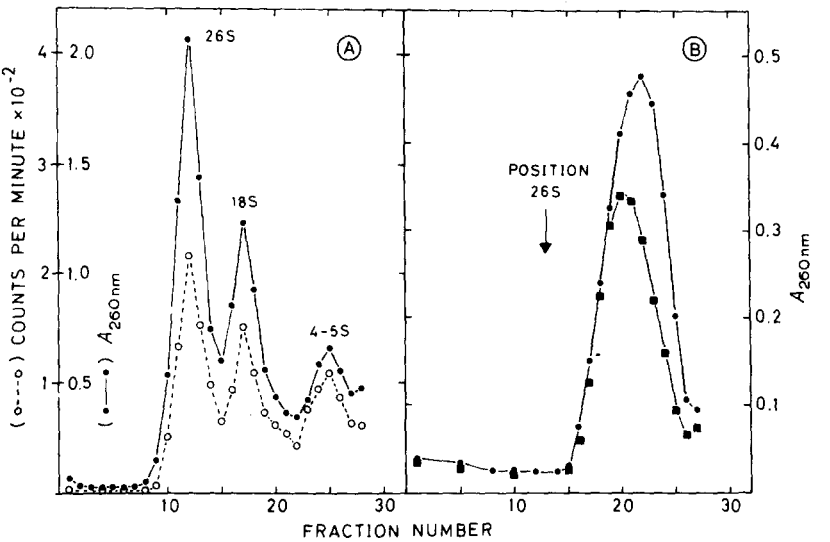

Fig. 4. RNA from *Drosophila* cells treated with ^{3}H-labeled 4′-aminomethyl trioxsalen and ultraviolet light. *Drosophila* cells ($2 \cdot 10^8$ cells $\cdot$ ml^{-1}) were incubated 60 min at 25°C with ^{3}H-labeled 4′-aminomethyl trioxsalen (154 μg $\cdot$ mol^{-1}, spec. act. $3.453 \cdot 10^{12}$ cpm $\cdot$ mol^{-1}). The suspension was frozen and irradiated while frozen for 9.50 h. RNA was extracted with phenol at 0°C, purified and centrifuged on a 5—20% sucrose gradient (A). 26 S and 18 S RNA was isolated from sucrose gradients, dissolved in 0.05 M Tris-HCl, pH 7.4, 10 mM EDTA, heated 5 min at 65°C and then re-centrifuged on sucrose gradients (B). •———•, heated 26 S RNA; ▪———▪, heated 18 S RNA.

13.6 under the same conditions. No psoralen molecules were bound without irradiation. Addition of 10% glycerol to the cell suspension before freezing did not change the number of 4′-aminomethyl trioxsalen molecules bound to DNA or RNA. Under the same conditions cell suspensions were incubated for 15, 30, 60 or 120 min and then irradiated for 4 h in the frozen state. Assuming 120 min of incubation gives maximal binding of 4′-aminomethyl trioxsalen to nucleic acids, 15 min of incubation gives 35% of maximal binding, 30 min gives 45% and 60 min of incubation gives 80% of maximal binding.

The number of 4′-aminomethyl trioxsalen molecules bound to RNA and DNA using different conditions of incubation and irradiation are shown in Table I. When *Drosophila* cells are incubated with relatively high concentrations of ^{3}H-labeled 4′-aminomethyl trioxsalen (118 μg $\cdot$ ml^{-1}) and irradiated in the frozen state, about 40 molecules of the psoralen are bound per 10^3 base pairs of DNA and about 20 molecules are bound per 10^3 base pairs of RNA. Under these conditions 15% of the drug is in the cellular fraction and 85% is in the medium. Increasing the concentration of ^{3}H-labeled 4′-aminomethyl trioxsalen in the medium does not increase the binding most likely because the uptake of drug cannot be further increased. Cells growing in the presence of ^{3}H-labeled 4′-aminomethyl trioxsalen for 4 h and 26 h show less binding of the drug to DNA than concentrated cell suspensions. Since the same ratio of drug to cells has been used in these experiments the results suggest that the extent of binding is dependent on the concentration of the drug in the medium and that the drug is not concentrated within the cells during cell growth. The efficiency of photoreaction in the frozen state was compared with that obtained in cell

TABLE I

BINDING OF ^{3}H-LABELED 4′-AMINOMETHYL TRIOXSALEN TO DNA AND RNA UNDER DIFFERENT CONDITIONS OF INCUBATION AND IRRADIATION

Cell type	Irradiation intensity ($mW \cdot cm^{-2}$)	Incubation time	Concentration in medium ($\mu g/ml$)	In cellular fraction (μg)	DNA (μg)	Mol bound per 10^3 base pairs	
						DNA	RNA
Drosophila cells (1)	3.2	4 h	18.5	—	32	15.0	—
Drosophila cels (1)	3.2	26 h	13.2	—	32	12.0	—
Drosophila cells (2)	3.2	30 min	118	18	40	33.4	14.6
Drosophila cells (2)	3.2	70 min	118	18	40	42.0	19.8
Drosophila cells (2)	3.2	90 min	205	—	20	37.8	—
Drosophila cells (3)	100	60 min	147	22	50	58.6	18.4
Drosophila nuclei (4)	100	30 min	26	—	20	69.6	—
Drosophila nuclei (4)	100	30 min	130	—	20	376.8	—
HeLa cells (5)	100	1 h	94	—	87	29.8	21.0

(1) *Drosophila* cells were grown in monolayer cultures with ^{3}H-labeled 4′-aminomethyl trioxsalen (spec. act. $2.0 \cdot 10^{12}$ cpm $\cdot$ mol^{-1}) for the time indicated. The cells were harvested, frozen and irradiated in the frozen state for 8 h.

(2) Cells were harvested, incubated with ^{3}H-labeled 4′-aminomethyl trioxsalen in a small volume of medium, frozen and irradiated in the frozen state for 8 h.

(3) As (2) but irradiated in suspension for 10 min at 0°C.

(4) *Drosophila* cells ($5 \cdot 10^{6}$) were homogenized in 2 ml 0.5% Nonidet P-40 in sodium citrate buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). $2 \cdot 15$ s on a Vortex mixer at 0°C, which disintegrates more than 99% of the cells. The nuclei were collected by centrifugation and incubated with ^{3}H-labeled 4′-aminomethyl trioxsalen (spec. act. $2.0 \cdot 10^{12}$ cpm $\cdot$ mol^{-1}) in 1.0 ml sodium citrate buffer at 0°C. Irradiation in suspension for 10 min at 0°C.

(5) HeLa cells ($6.3 \cdot 10^{7}$) grown in suspension were collected by centrifugation and incubated in 5 ml medium with ^{3}H-labeled 4′-aminomethyl trioxsalen. Irradiation in suspension for 10 min at 0°C.

suspensions and high intensity irradiation. In both cases 18—20 molecules of 4′-aminomethyl trioxsalen are bound to 10^3 base pairs in RNA but the number bound to 10^3 base pairs in DNA is increased from 42 to 58 molecules. When nuclei from *Drosophila* cells are incubated with concentrations of 4′-aminomethyl trioxsalen which corresponded to those used for whole cell incubation, 376 molecules of the psoralen are bound per 10^3 base pairs of DNA which is about 10 times higher than the number bound in whole cells. A five times lower concentration of 4′-aminomethyl trioxsalen in the incubation mixture still gives about 20% higher binding to DNA in nuclei than that obtained with whole cells (Table I). These results show that the nuclear membrane and the chromosomal proteins are of little hindrance for the reaction of 4′-aminomethyl trioxsalen with DNA. The binding of 4′-aminomethyl trioxsalen to DNA in whole cells seems to be limited by the amount of psoralen which can be taken up by the cells. When HeLa cells are incubated with 4′-aminomethyl trioxsalen (94 $\mu g \cdot ml^{-1}$) about 30 molecules of psoralen are bound per 10^3 base pairs in DNA and 21 per 10^3 base pairs in RNA (Table I).

The effect of 4′-aminomethyl trioxsalen binding to DNA in *Drosophila* cells was followed in the electron microscope. DNA with 32 molecules of 4′-aminomethyl trioxsalen bound per 10^3 base pairs was denatured in formamide and formaldehyde and spread for electron microscopy [37]. This treatment gives rise to looped DNA molecules kept together only by interstrand crosslinks of 4′-aminomethyl trioxsalen. An example is seen in Fig. 3A. The size of the loops are highly heterogenous at all degrees of crosslinking investigated. In DNA having 32 molecules of 4′-aminomethyl trioxsalen bound per 10^3 base pair about 20—30% of the DNA is double stranded and when the size of 450 loops were measured the sizes varies from 100 base pairs to 800 base pairs without any regular 200 base pairs pattern as has been demonstrated in the reaction of trioxsalen with chromatin [13,30]. The very heavy reaction of 4′-aminomethyl trioxsalen with DNA in isolated nuclei (Table I) is confirmed by electron microscopy. DNA molecules containing 70 molecules of 4′-aminomethyl trioxsalen bound per 10^3 base pairs shows very long stretches of double stranded DNA and in DNA molecules with 376 molecules bound the DNA is almost completely double stranded. The DNA molecule shown in Fig. 4B contain more loops than the average.

In *Drosophila* cells the mature ribosomes contain a 26 S RNA with a nick almost in the center of the molecule [43,44]. When isolated 26 S RNA is treated with 4′-hydroxymethyl 4,5′,8-trimethyl psoralen and ultraviolet light the two fragments are held together by covalent crosslink in a 200 base pair hairpin in the middle of the molecule [45]. When isolated 18 S RNA from *Drosophila* cells is treated similarly a loop is found in one end of the molecule [45]. In order to demonstrate such structures within the ribosomes *Drosophila* cells were treated with 4′-aminomethyl trioxsalen and ultraviolet light as described in Fig. 4. RNA was extracted and separated into 26 S, 18 S and 4—5 S RNA peaks by sucrose gradient centrifugation (Fig. 4A). 26 S and 18 S RNA was isolated, heated 5 min at 65°C and re-run in sucrose gradient. Although the RNA is heavily reacted with the drug the absence of a 26 S peak demonstrates that the two pieces are not bound together by crosslinks (Fig. 4B). The peak from 26 S RNA is broader than the 18 S peak because

26 S RNA consists of two pieces of unequal length [45] and a 5.8 S and 2 S RNA [46]. No loops or hairpins were observed when 26 S or 18 S RNA was examined under denaturing conditions in the electron microscope. This lack of characteristic structures holds also for ribosomal RNA from HeLa cells treated with 4′-aminomethyl trioxsalen and ultraviolet light as described in Table I.

The increased RNAase activity in 4′-aminomethyl trioxsalen treated cells suggests that this compound react with proteins or membrane components in the lysosomes causing a breakdown of this organelle and a release of degradative enzymes. The binding of 4′-aminomethyl trioxsalen to cellular proteins was studied in *Drosophila* cells. Cells were incubated with the tritiated drug. Half of the cell suspension was irradiated as described in Table I, Expt. 3 and the other half was not irradiated. A similar batch of cells was mixed with tritium labelled 4′-aminomethyl trioxsalen which had been irradiated for the same length of time before mixing with the cells. The cells were added to ice-cold perchloric acid and then taken through a modified Smith-Tannhauser washing procedure [39]. In the three experiments 3—4% of the added ^{3}H-labeled 4′-aminomethyl trioxsalen was found in the protein fraction and this is about 5—10 times more than is found in the DNA fraction. About 90% of the radioactivity is removed during the initial washings with perchloric acid, alcohol. The washing procedure involves heating in alcohol-ether to 65°C, digestion in 0.3 M KOH at 37°C for 17 h and treatment with 0.25 M perchloric acid at 95°C. Dissolving the residual protein fraction once more in KOH and re-precipitation with perchloric acid removes about 10% of the protein bound ^{3}H-labeled 4′-aminomethyl trioxsalen. The strong binding of this psoralen derivative and its photodecomposition products is apparently not due to covalent bonds and not dependent on a photoreaction. *Drosophila* cells were therefore incubated with ^{3}H-labeled 4′-aminomethyl trioxsalen at different temperatures and time periods and then without irradiation taken through the Smith-Tannhauser washing procedure. The same large amount of ^{3}H-labeled 4′-aminomethyl trioxsalen was found in the protein fraction whether cells were incubated at 25°C for 15 min or 30 min or at 0°C for 15 min or just mixed, centrifuged and processed. The strong binding of ^{3}H-labeled 4′-aminomethyl trioxsalen to proteins was also seen after phenol extractions of cells treated with the drug and irradiated as described in Table 1, Expt. 2. Phenol is a very potent solvent for ^{3}H-labeled 4′-aminomethyl trioxsalen and its photodecomposition products. When no more tritium labelling was removed by repeated washings with phenol and 70% alcohol, treatment with pronase allows 60% of the label to pass through a dialysis bag leaving the remaining 40% bound mainly to DNA.

Discussion

4′-aminomethyl trioxsalen is a new psoralen derivative which has a 13 000 times higher solubility than trioxalen and a 200 times higher solubility than 8-methoxypsoralen [26] in water. 4′-aminomethyl trioxsalen is therefore well suited for whole cell experiments because the drug may be administered in sufficiently high concentration to give a high degree of photoreaction after one single addition followed by irradiation with ultraviolet light. This advantage is found to be important because treatment of different cells with 4′-amino-

methyl trioxsalen and ultraviolet light gives rise to the release of lysomal enzymes which in time results in heavily degraded RNA. In order to prevent the degradation the cells are incubated with 4′-aminomethyl trioxsalen and then frozen quickly to minus 78°C and the cells are then irradiated in the frozen state. By this technique it is possible to obtain a high degree of photoreaction with both DNA and RNA and avoid degradation.

4′-aminomethyl trioxsalen reacts with ribosomal RNA and tRNA in HeLa cells and *Drosophila* cells. Using tritiated 4′-aminomethyl trioxsalen the specific activity of 26 S, 18 S and 4—5 S RNA is found not to be significantly different. The strong binding of 4′-aminomethyl trioxsalen to nucleic acids is probably due to the positively charged side group.

The 26 S ribosomal RNA from *Drosophila* cells contain a 200 base pair hairpin near the middle of the molecule when the isolated RNA is kept at helix stabilizing conditions. This helix has been stabilized by psoralen photo-crosslinking the two pieces and in a similar manner the 18 S RNA has been demonstrated to contain a loop in one end of the molecule [45]. When ribosomal RNA was extracted from *Drosophila* cells treated with 4′-aminomethyl trioxsalen and irradiated in the frozen state no secondary structure could be seen. Ribosomal RNA from treated HeLa cells also revealed no secondary structure.

It is possible that 4′-aminomethyl trioxsalen is covalently bound to proteins after irradiation but this could not be proven from these experiments. It was found that ^{3}H-labeled 4′-aminomethyl trioxsalen as well as its photodecomposition products bind strongly to the protein fraction even in the absence of photoexcitation. The amount bound to proteins is more than that bound to DNA. This strong adsorption of 4′-aminomethyl trioxsalen to proteins is not a special property of this psoralen derivative due to the 4′-aminomethyl group. Several years ago it was found that trioxsalen binds to proteins in the epidermis cells [25]. 8-methoxypsoralen and its photodecomposition products have been shown to adsorb to some proteins so strongly that the compounds could not be removed by gel filtration [16,20,25] or by electrophoresis in sodium dodecyl sulphate buffer [42]. It is possible that 4′-aminomethyl trioxsalen is bound to proteins or lipids in the lysosomal membrane causing rupture of the lysosomes. Such à mechanism would be in accordance with the effect described for anthracene and other polybenzoid hydrocarbons. These molecules are taken up by the lysosomes and after irradiation there is a release of degradative enzymes and an increase in chromosome aberations [40,41]. Treatment of human lymphocytes in vitro with trioxsalen or 8-methoxypsoralen and ultraviolet light also induces chromosome damage [47] and human fibroblast cells treated in culture show an increase in proteolytic enzymes in the cell-free supernatant [42]. These effects of psoralens and ultraviolet light suggest an effect via the lysosomes. The effect of ultraviolet light on lysosomes is well described [48, 49] and the importance of lysosomes in psoriasis treatment has been discussed but most emphasis has been laid on the crosslinking effect of psoralens. The failure to find a significant increase of crosslink in DNA from the psoriatic skin of patients treated with 8-methoxypsoralen and ultraviolet light [50] suggest, however, that the role of the lysosomes should be studied more closely.

The extremely strong binding of 4′-aminomethyl trioxsalen to nucleic acids

makes this compound an excellent tool for the study of nucleic acid structures in vivo and since the effect of this compound plus ultraviolet light on the lysosomes is pronounced this psoralen may be a useful tool for in vivo studies of lysosomal disruption as well.

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