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DNA-Protein Cross-Linking by Chemical Carcinogens in Mammalian Cells[1]

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ABSTRACT

The induction of DNA cross-linking in mammalian cells by various carcinogens was investigated by the method of alkaline elution. A dose-dependent increase in DNA cross-linking was seen following exposure of human fibroblasts to *N*-acetoxy-2-acetylaminofluorene and following exposure of mouse embryo cells to 7,12-dimethylbenz[a]-anthracene. No cross-link effect was seen following treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, benz-[a]anthracene, benz[a]anthracene-5,6-dihydroepoxide, or metabolic inhibitors. The cross-linking appeared to be DNA-protein in nature since proteinase treatment removed the effect. DNA single-strand breaks were also induced by several of these agents. In the case of *N*-acetoxy-2-acetylaminofluorene and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, approximately 70 to 90% of these breaks were rejoined after an 18-hr incubation in fresh medium, whereas repair of the cross-links induced by *N*-acetoxy-2-acetylaminofluorene was slight at this time.

INTRODUCTION

The binding of chemical carcinogens or their metabolically activated products to DNA is believed to be an essential part of the carcinogenic process (21). Most of these agents have been shown to be mutagenic in bacterial systems (2). The possibility exists that certain potent carcinogens may owe their activity to the formation of covalent cross-links between a DNA strand and adjacent macromolecules. Although often more cytotoxic on a molar basis, there does not appear to be a concomitant increase in the mutation rate when the effects of polyfunctional agents are compared to those of monofunctional agents (28). However, unlike most monofunctional alkylating agents, polyfunctional agents are inducers of frame-shift mutations, as are many potent chemical carcinogens (2). In patients receiving polyfunctional alkylating agent therapy, second neoplasms have appeared in organs exposed to high concentrations of the active agent; in several instances, the latent periods have been surprisingly short (29, 32, 34).

When mammalian cells were treated with certain chemical carcinogens (12), the extractability of DNA from protein was diminished. However, the interpretation that this effect was specific for DNA-protein cross-links was weakened when a similar effect was seen with various metabolic inhibitors (12). Evidence has been presented that DNA-protein cross-links are formed *in vitro* when high ratios of carcinogen to DNA and protein are incubated in solution; this effect was seen with nitrogen mustard, N-AcO-AAF,[4] and MNNG among others (18, 21). The present study was undertaken with N-AcO-AAF, DMBA, and MNNG as possible inducers of DNA cross-linking.

When a high concentration of N-AcO-AAF (8 mol carcinogen:1 mol DNA base pairs) is incubated with DNA in solution, DNA-DNA cross-links between complementary strands of DNA were seen (20). Similar cross-links were observed (11) when >5% of the bases in purified DNA were modified by N-AcO-AAF, although these cross-links were sensitive to alkali (pH 9) or high temperature. When methyl groups are added at the 7 and 12 positions of BA to form DMBA, there is a progressive increase in the relative carcinogenicity (28). The possibility exists that with the activation of multiple reactive side groups, DMBA may function as a cross-linking agent.

Extensive evidence has been presented that the technique of alkaline elution can detect DNA SSB produced by X-rays (7, 15, 16) and various chemical agents (5, 14, 30) and by excision breaks produced after UV radiation (8). The advantage of this technique is the sensitivity to detect extremely low levels of damage. It can also quantitatively measure DNA cross-linking by the reduction in elution rate of sheared DNA when the cells are first treated with a cross-linking agent (6, 7, 9, 27). DNA-protein cross-links can be distinguished from presumed DNA-DNA cross-links by the sensitivity of the former to proteinase digestion. In the present investigation, DNA-protein cross-links were detected with this technique after treatment of mammalian cells with N-AcO-AAF or DMBA. No such cross-linking was induced by MNNG, BA, BA-epoxide, or various metabolic inhibitors. SSB were produced by several of these agents; evidence is presented for the possible repair of both SSB and cross-links.

MATERIALS AND METHODS

Cells and Cell Labeling. The fibroblast cell strain CRL 1220 (American Type Culture Collection, Rockville, Md.) was derived from a human adult donor and used at passages 5 to 23 (1:2 split). The cells were grown and prepared for experiments as previously described (7). A mouse embryo fibroblast cell line ($10T^1/_2$ clone 8) was grown as previously described (31); stock cultures were maintained in 60-mm Petri dishes and passaged every 7 days by a 1:20 dilution. Cells were used at passages 8 to 15. [2-^{14}C]Thymidine was added to growing human or mouse cells for several days and then removed. On the day of the experiments, the cells were in a confluent monolayer.

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[4] The abbreviations used are: N-AcO-AAF, *N*-acetoxy-2-acetylaminofluorene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DMBA, 7,12-dimethylbenz[a]anthracene; BA, benz[a]anthracene; BA-epoxide, benz[a]anthracene-5,6-dihydroepoxide; SSB, single-strand breaks.

Exponentially growing L1210 mouse leukemia cells were labeled with [^{3}H]thymidine as previously described (7).

Irradiation. X-rays were delivered by a GE Maximar X-ray generator operating at 220 kV and yielding a dose rate of 80 rads/min. The cells were irradiated at 0° suspended in medium as previously described (9).

Chemical Agents. BA, DMBA, and BA-epoxide were kindly supplied by the Information and Resources Segment, Carcinogenesis Program, Division of Cancer Cause and Prevention, National Cancer Institute, from the Chemical Carcinogen Repository, IIT Research Institute. N-AcO-AAF was originally supplied by J. Miller, McArdle Laboratory for Cancer Research, Madison, Wis.; and MNNG, by the Aldrich Chemical Co., Milwaukee, Wis. These agents were stored at −120° and dissolved in acetone (BA, DMBA), dimethyl sulfoxide (BA-epoxide), or 10% dimethyl sulfoxide in alcohol (N-AcO-AAF) immediately prior to the experiments. When fibroblasts were treated with these agents, the stock solution was added directly to the cells and medium such that the final concentration of solvent was 0.2%. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.), sodium arsenite, and KCN were dissolved directly in medium immediately prior to the experiment. At the end of this treatment, the medium was removed and the monolayer was rinsed once with medium and 3 times with 0.9% NaCl solution and then suspended in cold medium as described previously (9). When incubation followed drug treatment, warm medium was added to the rinsed monolayer for the indicated times prior to suspension in cold medium. The cells were shielded from light when possible.

Alkaline Elution. The alkaline elution procedure used is a modification of that described by Kohn and Ewig (16). Briefly, the cells were filtered onto a polyvinyl chloride filter, lysed with 2 M NaCl, 0.02 M trisodium EDTA:0.2% Sarkosyl (pH 10.2), washed with 0.02 M trisodium EDTA (pH 10.3), and then eluted at 0.04 ml/min with a solution consisting of 0.02 M EDTA (acid form) plus tetrapropylammonium hydroxide (40% in H_2O; RSA Co., Ardsley, N. Y.) added in the amount required to give a pH of 12.2. Eluted fractions were collected and assayed for radioactivity as previously described (7). To provide for an internal standard, ^{3}H-labeled L1210 cells which had received 150 rads at 0° were included in each assay. To improve quantification, elution of sample DNA has been plotted against the elution of the internal reference DNA; there is little change in the shape of the elution curves since the internal reference DNA elutes in a nearly linear fashion. Since the internal reference DNA elutes at a constant rate on a logarithmic plot, the hr of elution have been corrected such that 50% of the internal reference cell DNA has eluted off the filter in 12 hr (9).

Evidence has been presented that the alkaline elution technique can quantitatively measure DNA SSB produced by low doses of X-rays (7, 8, 15) as measured by the relative retention (fraction of DNA retained on the filter after 12 hr). Assuming a constant efficiency for DNA single-strand breakage (19), an estimate can then be made of the average DNA strand size after various low doses of X-ray or after treatment with agents producing comparable levels of single-strand breakage (14). Treatment of the cells with a cross-linking agent, however, diminishes the effect of subsequent X-irradiation on alkaline elution; hence, the number of SSB produced by the subsequent X-rays (9, 15) or the agent itself (5, 7, 9) will be underestimated. This cross-link effect, seen by alkaline elution, has been quantified by comparing the increase in elution produced by a small test dose of X-rays in treated cells to the increase by the same test dose of X-rays on untreated cells (9). This ratio has been termed the cross-link factor:

$$\frac{\text{Mean log (relative retention, no X-rays)} - \text{log (relative retention, X-rays)}}{\text{Log (relative retention, treated, no X-rays)} - \text{log (relative retention, treated, X-rays)}}$$

With no cross-linking effect, this parameter is 1 by definition. When cells are treated with various DNA-DNA cross-linking agents (nitrogen mustard, mitomycin C) or DNA-protein cross-linking agents (UV or high-dose X-rays), the cross-link factor increases in a manner approximately directly proportional to the dose of the cross-linking agent (9).

In some experiments, the cell lysates were digested with proteinase prior to elution (6); this treatment is a modification of that previously described (7, 9). Proteinase-K (EM Laboratories, Inc., Elmsford, N. Y.), dissolved in the lysing solution described above to a concentration of 0.5 mg/ml, was added to the cells on the filter instead of the regular lysing solution and allowed to incubate at room temperature for 1 hr. This solution was then allowed to drain through the filter; the filter was next washed with 0.02 M trisodium EDTA (pH 10.3), and the alkaline elution was carried out as described previously (6, 7, 9).

RESULTS

In Chart 1*A*, the effect of a 300-rad test dose of X-rays is shown. Most of the DNA was retained on the filter from the control cells, whereas with X-rays (×) the elution rate was increased. When cells were treated with N-AcO-AAF, the elution rate also increased, indicating that this carcinogen induces DNA SSB as measured by alkaline elution at pH 12.2. When these carcinogen-treated cells were subsequently exposed to the same test dose of X-ray (N-AcO-AAF + ×), elution increased somewhat, but in a nonlinear fashion characteristic of cells treated with a cross-linking agent (9). At 12 hr of elution, almost as much DNA is retained on the filter in the sample treated with the carcinogen plus X-rays as in the sample receiving X-rays alone, despite the fact that the carcinogen alone induced a significant increase in elution. This diminished effect with a test dose of X-rays was not seen when the cells were treated with a monofunctional agent which induced DNA SSB only (9).

This cross-link effect was also seen after exposure of cells to DMBA. Here, metabolic activation of the carcinogen is required (28); mouse C3H/10T½ cells were used which have been demonstrated to activate polycyclic aromatic hydrocarbons (25). The effect of X-rays alone on alkaline elution did not differ significantly between the human and mouse cell strains. The results are presented in Chart 2*A*; the effect with DMBA was similar to that seen with N-AcO-

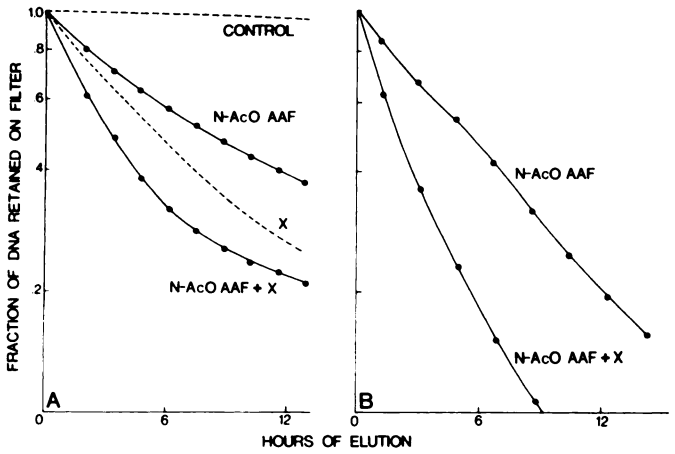

Chart 1. Effect of cell treatment with N-AcO-AAF on alkaline elution. *A*. Human fibroblasts were treated with 10 μM N-AcO-AAF for 1 hr at 37°, suspended in cold medium, X-irradiated with 300 rads at 0° where indicated (×), and analyzed by alkaline elution. Elution profiles of untreated cells (*control*) and cells exposed only to 300 rads at 0° (×) are also included. In *B*, treatment was the same as in *A*, except that cell lysates were digested with proteinase-K prior to alkaline elution.

AAF. Incubation of the cells with DMBA induced DNA SSB, as reflected by an increase in the elution rate. Again, the effect of subsequent irradiation with a small test dose of X-rays was reduced in carcinogen-pretreated cells. As can be seen in Chart 2*A*, after 12 hr of elution, more DNA was retained on the filter in the sample treated with DMBA and X-ray than on that treated with X-ray alone.

A measure of the amount of DNA cross-linking as determined by the cross-link factor is shown for both N-AcO-AAF and DMBA (Chart 3). A 1-hr incubation of the cells with the proximate carcinogen N-AcO-AAF produced a dose-dependent increase in the cross-link factor (Chart 3*A*). The relative retention [fraction of DNA retained on the filter after 12 hr of elution (Chart 3*A*)] decreased with increasing dose to 10 μM N-AcO-AAF and then appeared to level off. The actual SSB frequency, however, would continue to increase at higher doses since an increasing number of the SSB are obscured by the increasing cross-link factor. Therefore, an estimate of the actual number of SSB cannot be made from the relative retention value without also considering the cross-link effect. With removal of the carcinogen and an 18-hr incubation in fresh medium, the relative retention approached control levels, indicating that most of the DNA SSB have been rejoined by this time. The cross-link factor, however, only slightly decreased during this time. It should be noted that there was no significant release of DNA into the medium during this period.

A similar effect is seen after treatment of mouse cells with DMBA for 4 hr and a subsequent 12-hr incubation in fresh medium (Chart 3*B*, *open circles*). With a 4-hr exposure of the cells to this carcinogen and no subsequent incubation (Chart 3*B*, *closed circles*), only a slight cross-link effect and a small decrease in the relative retention occurred which was not dose dependent. One can speculate that the activation of DMBA to the ultimate carcinogen(s) which produces DNA damage may be a relatively slow process in these cells. It should be noted that DNA damage, as reflected by the above parameters, was somewhat less in experiments in which a different lot of serum was used. To produce the same effect as seen in Chart 3*B* required more prolonged exposures to DMBA with this serum lot (*e.g.*, 20 hr of exposure in Chart 2*A*). No diminution in these parameters was seen in experiments with agents not requiring metabolic activation (N-AcO-AAF, MNNG, X-rays) at any time with several lots of serum.

When the cell lysates were digested with proteinase-K prior to alkaline elution, the cross-linking effect induced by the presumed DNA-protein cross-linking agents, UV irradiation (7), or high-dose X-rays (9) was removed. On the other

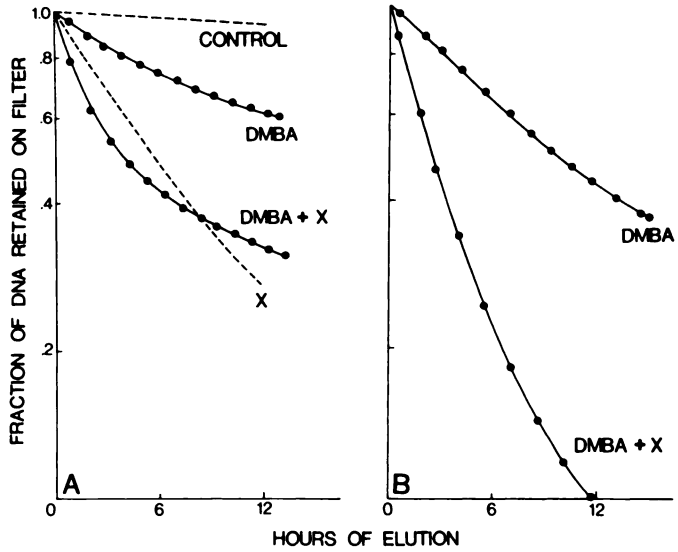

Chart 2. Effect of cell treatment with DMBA on alkaline elution. *A*. Mouse C3H/10T½ cells were treated with 10 μM DMBA for 20 hr at 37°; the cells were then analyzed as in Chart 1; *control* and × curves are defined in Chart 1. *B*, with proteinase-K digestion.

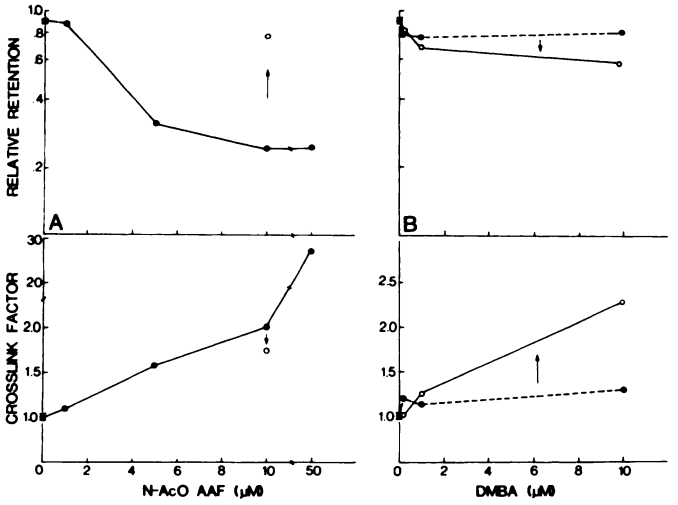

Chart 3. Strand break effect (relative retention) and cross-link effect (cross-link factor) of N-AcO-AAF and DMBA. *A*. The relative retention (fraction of DNA retained on the filter after 12 hr of elution) and the cross-link factor (see "Results") were determined after human cells were treated with N-AcO-AAF as in Chart 1. O with *arrow*, effect of 18 hr of incubation in fresh medium at 37° after removal of the carcinogen. *B*. The same parameters were determined after incubation of mouse cells for 4 hr with DMBA (●) or after an additional 12 hr of incubation in fresh medium (○) as described in Chart 2. Mean ± S.E. for controls are included: human cells, 17 determinations; mouse cells, 15 determinations.

hand, the sensitivity to low-dose X-ray as measured by the relative retention was not markedly changed (9). As can be seen in Charts 1*B* and 2*B*, the cross-link effect induced by N-AcO-AAF and DMBA was also removed with proteinase digestion. No significant cross-linking remained after proteinase digestion at these doses. With carcinogen treatment alone, the elution of DNA from the filter was increased, and the effect of subsequent X-irradiation was also increased. These data indicate that the predominant type of cross-linking which occurs with these 2 agents is DNA-protein or at least mediated by one or more proteinase-sensitive sites.

The effect of cell treatment with MNNG on alkaline elution is demonstrated in Chart 4. Here, the relative retention decreased in a near-first-order relation to the dose of carcinogen used. Since no significant cross-linking effect was seen with MNNG (see Table 1), the effect of MNNG can be compared directly to the effect of X-rays; *e.g.*, treatment of the cells with a 1-μM dose of MNNG for 1 hr produces the same SSB effect, as measured by the relative retention, as a dose of approximately 225 rads of X-rays. After 18 hr of repair incubation, many of the SSB measured by alkaline elution have been rejoined, with approximately 75% recovery after 2.5 μM MNNG. It should be noted that the elution profiles of DNA from cells treated with MNNG were linear for the first 8 to 10 hr of elution, indicative of a random distribution of SSB (15); but there was a late, moderate acceleration of the elution rate, suggestive of continued SSB production after longer exposure to pH 12.2. This effect has been previously seen with the alkylating agent 1-methyl-1-nitrosourea (6) and was attributed to alkaline-labile sites in the DNA.

Since certain metabolic inhibitors, as well as known DNA-DNA cross-linking agents, decreased the DNA extractability from protein (12), the effect of cell treatment with metabolic inhibitors was examined to determine the specificity of DNA cross-linking as measured by alkaline elution. These results are shown in Table 1. After a 1-hr exposure of mouse cells to toxic doses of sodium arsenite, no cross-linking effect was seen; it should be noted that sodium arsenite induced a moderate level of SSB (relative retention equivalent to 120 rads of X-rays). No SSB were seen after a 1-hr exposure to KCN; after a 24-hr exposure to KCN, when the cells showed marked lytic changes microscopically with <20% of cells remaining attached, the relative retention did decrease, but again no cross-linking effect was detected (data not shown). Treatment of both human and mouse cells with the protein synthesis inhibitor cycloheximide produced no significant cross-linking or SSB effect. It should be noted that neither cycloheximide nor a 1-hr exposure to KCN affected clonogenic survival in confluent cultures. The effect of MNNG and BA-epoxide is also illustrated in Table 1; both these agents are seen to induce SSB as detected by a decrease in the relative retention, but no significant cross-linking effect was observed. No SSB or cross-link effects were seen after a 20-hr exposure to 100 μM BA. In fact, no effect was seen with BA in doses varying from 5 to 500 μM and incubation times of 4 to 24 hr. Caution must be taken in interpreting these results, however, since BA had only a slight effect on cell survival in these experiments.

To exclude a direct DNA-carcinogen filter adherence, DNA was incubated with N-AcO-AAF and then diluted with eluting solution and slowly passed through the filter. Purified *Escherichia coli* [^{14}C]DNA (2.5 μg; New England Nu-

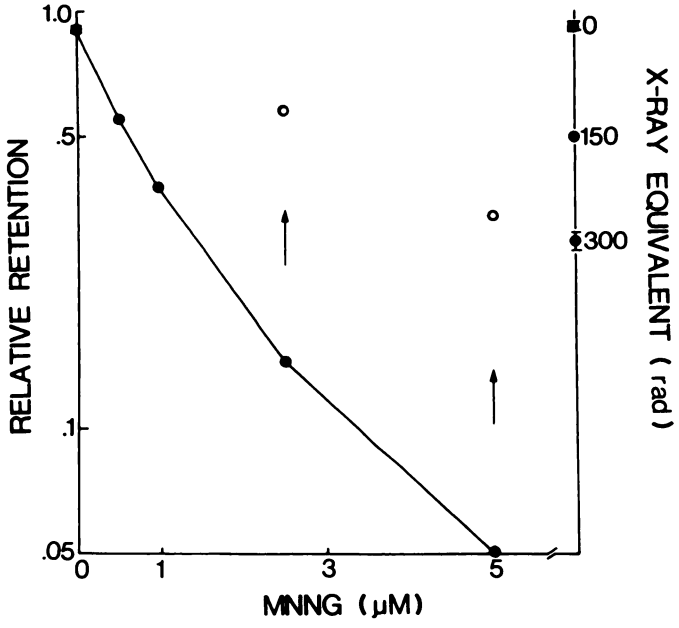

Chart 4. Effect of MNNG on relative retention of DNA. Human cells were treated with MNNG for 1 hr (●) or rinsed and incubated with fresh medium for an additional 18 hr (○). *Right abscissa*, effect of X-rays alone, shown for comparative purposes.

Table 1

Effect of cell treatment with metabolic inhibitors and other agents on alkaline elution

The relative retention and the cross-link factor were derived as before (see "Results"); the test dose of X-rays was 300 rads when determining the cross-link factor with these agents. Following incubation with these agents at 37°, confluent cultures of mouse cells were trypsinized and replated at the appropriate densities, and clonogenic survival was determined as previously described (33). Plating efficiency of controls with or without 0.2% solvent (acetone) was 21%.

	Relative retention	Cross-link factor	% of survival (S/S_0)
Mouse C3H/10T½ fibroblasts			
Control (15 determinations)	0.91 ± 0.01[a]	1.00 ± 0.02[a]	
Sodium arsenite (1 mM, 1 hr)	0.56	0.96	2.2
KCN (1 mM, 1 hr)	0.93	0.98	99
Cycloheximide (10 μg/ml, 24 hr)	0.94	1.04	100
BA (100 μM, 20 hr)	0.91	1.02	81
Human fibroblasts			
Control (24 determinations)	0.90 ± 0.01[a]	1.00 ± 0.03[a]	
MMNG (1 μM, 1 hr)	0.49	1.09	
MNNG (2.5 μM, 1 hr; 18-hr drug-free medium)	0.58	1.07	
Cycloheximide (10 μg/ml, 24 hr)	0.93	1.1	
BA-epoxide (100 μM, 1 hr)	0.54	0.94	

[a] Mean ± S.E.

clear, Boston, Mass.) was incubated in 1 ml of buffered 0.9% NaCl solution and 0.01 ml of solvent or N-AcO-AAF (final concentration, 1 or 100 μM) for 1 hr at 37°. The solution was then diluted with 20 ml of the standard eluting solution and pumped through the filter at 0.04 ml/min. Neither concentration of carcinogen had a significant effect on elution. N-AcO-AAF did not retard the passage of DNA through the filter at any time.

DISCUSSION

When DNA SSB are produced by a small test dose of X-rays, the increased elution of DNA from the filter is diminished if the cells have previously been treated with a cross-linking agent (7, 9, 15). In the present investigation, this effect was produced by treatment of cells with N-AcO-AAF or DMBA. A dose-dependent increase in DNA cross-linking was seen with increasing concentration of N-AcO-AAF; a similar effect was seen with DMBA after longer exposure to cells which can metabolically activate this agent. As has been found with previously studied DNA-protein cross-linking agents (7, 9), treatment of the cell lysates with proteinase prior to alkaline elution removed the cross-linking effect induced by N-AcO-AAF and DMBA. This finding would imply that most cross-linking detected is DNA-protein in nature. This cross-linking is resistant to salt (2 M NaCl), detergent, and long exposure to alkali, implying a rather tight, stable adherence. After incubation of cells treated with N-AcO-AAF in fresh medium, there was evidence of only minimal repair of cross-links, with approximately 70% of the cross-linking effect remaining after 18 hr of incubation. No significant cross-linking effect was seen after incubation of cells with MNNG, BA, BA-epoxide, sodium arsenite, KCN, or cycloheximide.

An estimate of the SSB and cross-link frequencies have been derived after cell treatment with several carcinogens in Table 2. In the case of MNNG, the SSB derivation is relatively simple since no cross-links are present. With N-AcO-AAF and DMBA, the SSB frequency can be derived after the cross-links have been removed with proteinase. Since the cross-link factor is actually an estimate of the factor by which the strand break effect (X-ray sensitivity) is reduced by cross-linking (9), the SSB frequency can also be derived without proteinase digestion. For example, in Chart 2*A*, DMBA induced an increase in elution equivalent to 100 rads of X-rays; since the cross-link factor is 1.89, the actual effect of DMBA would be equivalent to 189 rads. This is in agreement with Chart 2*B* where an effect equivalent to approximately 180 rads was seen after proteinase treatment.

If the cross-linking effect seen with alkaline elution after N-AcO-AAF or DMBA treatment does represent a tight binding of DNA to protein, several possibilities can be proposed to explain this effect. The most attractive would be that these agents act as bifunctional agents with resultant cross-linking. A more simple explanation would be a direct DNA-carcinogen filter adherence; this seems unlikely, however, since the cross-linking is proteinase sensitive and since treatment of DNA *in vitro* with high concentrations of N-AcO-AAF (molar ratio of up to 10^4 N-AcO-AAF molecules:1 DNA base pair) resulted in no adherence of DNA to the filter. When methylmethanesulfonate-treated DNA is heated to 50°, DNA-DNA cross-links are produced in low yield, which are proportional to the rate of depurination (3). The possibility exists that a similar degradative phenomenon has occurred here, but it would appear unlikely that with only 1 hr of exposure to relatively low doses of N-AcO-AAF at 37° that this effect would be a significant one.

Table 2

Estimate of strand break and cross-link frequencies after exposure to chemical carcinogens

X-ray equivalent is the X-ray dose which resulted in the same relative retention as the carcinogen. The average number of SSB/cell was derived by assuming a constant efficiency (19) for strand breakage by X-rays. The X-ray equivalent corrected for cross-linking was derived in a similar manner but after proteinase digestion, which removed the cross-link effect. It was also derived from the product of the X-ray equivalent and the cross-link factor (see "Discussion"); both methods gave similar results. It has been estimated that 3100 DNA-protein cross-links with a cross-link factor of 2.0 result from exposure to a high dose of x-rays (9) (this is within a factor of 2 of that made by Kohn by a different approach[a]). Since the cross-link factor is approximately directly proportional to the dose of the cross-linking agent (9), one can estimate the actual number of DNA-protein cross-links if one assumes that these cross-links have the same effect on elution as those produced by high-dose X-rays. Diploid human fibroblasts were exposed to N-AcO-AAF or MNNG for 1 hr and tetraploid C3H/10T½ mouse cells were exposed to DMBA followed by long posttreatment incubations (as in Charts 2 and 3*B*, *open circles*). The SSB/cross-link ratio was derived from results in individual experiments.

	X-ray equivalent (rads)	Cross-link factor	X-ray equivalent corrected for cross-linking (rads)	SSB/cell	Cross-links/cell	SSB/Cross-link
MMNG (1 μM)	225			1700		
AAF (10 μM)	210–330	2.0–2.6	400–660	3100–5200	3100–4100	1.0–1.7
DMBA (10 μM)	100–110	1.9–2.3	18–230	2300–3000	4600–5600	0.5

[a] K. W. Kohn, personal communication.

Recently, an untwisting enzyme has been described in bacteria, which both nicks the DNA single strand and covalently attaches to one of the ends of the broken strands (4). The possibility exists that some repair-type enzyme may be producing similar DNA-protein cross-links after damage by N-AcO-AAF or DMBA. In support of this hypothesis is the observation that the ratio of SSB:cross-links is close to 1 for both agents (Table 2). Kohn *et al.* (17) have implicated this type of mechanism in the repair of DNA damage by intercalating agents, but unlike N-AcO-AAF and DMBA, these presumed endonuclease-induced SSB were preferentially masked by the adjacent protein during alkaline elution. In the present study, SSB induced by the carcinogens or subsequent X-ray exposure were masked to the same degree by the DNA-protein cross-linking effect. Also arguing against this interpretation is the observation that with 18 hr of repair of N-AcO-AAF damage (Chart 3), approximately 10% of the SSB remain, whereas 70% of the cross-links remain. A final objection may be that the cross-link effect may reflect cell lethality, although the data in Table 1 indicate that this is not the case. Significant DNA-protein cross-linking was detected after exposure to 10 μM N-AcO-AAF; clonogenic survival after this dose has been reported to be 10% (1). The same level of DNA cross-linking by alkaline elution (9) is also seen following treatment of human fibroblasts with either UV radiation or mitomycin C at doses which also yield about 5 to 10% clonogenic survival (10, 33). MNNG, however, produced no cross-linking effect following a 1-hr treatment with 2.5 μM, whereas 10% survival has been reported in human cells (HeLa) incubated for 1 hr with only 0.1 μM (26).

If N-AcO-AAF and DMBA do indeed induce DNA-protein cross-links, the molecular mechanisms which produce this effect are unknown. As previously stated, evidence has been presented that N-AcO-AAF induces cross-links between DNA and adjacent molecules *in vitro* at high molar ratios of N-AcO-AAF:DNA (11, 20, 22). If this carcinogen does become a bifunctional adduct, it must be with a rather low efficiency since approximately 10^6 molecules of N-AcO-AAF per cell genome are bound (1) at the same dose which induces 3000 to 4000 DNA-protein cross-links as detected by alkaline elution. Reactive epoxide groups can be formed at several positions on the DMBA molecule via metabolic activation (28). It is tempting to speculate that some molecules may be activated at multiple positions prior to or during reaction with DNA and adjacent nucleoproteins. The function of the methyl groups at the 7 and 12 positions remains unclear. If they are metabolically activated in DMBA to form a proximate carcinogen, the methyl group(s) may contribute to the cross-linking ability of DMBA. The effect of DNA-protein cross-links on malignant transformation remains to be determined. However, cross-linking was induced by DMBA and N-AcO-AAF but not by the less carcinogenic BA and BA-epoxide. It was also not induced by MNNG, but the cellular effects of this agent may be explained by the fact that MNNG produces a several hundred-fold greater number of alkylations than do similar concentrations of other monofunctional alkylating agents (26) or that MNNG may alkylate preferentially at critical sites.

As mentioned earlier, SSB were detected by alkaline elution after treatment of cells with several carcinogens. In the case of N-AcO-AAF, a low level of SSB was produced comparable to that seen after equally toxic doses of UV radiation (8). Since N-AcO-AAF damage, like UV radiation damage, is repaired by a "long-patch" excision pathway (24), the SSB detected may well represent transient endonuclease-induced strand breaks. With metabolic inhibitors, some SSB were seen at toxic doses. The cause for this effect may be a function of cell death with resultant release of endonucleases since DNA degradation has been reported in dying cells (14). In the case of MNNG, SSB were produced in a dose-dependent manner, as has been previously reported (23, 30). Several possible mechanisms have been proposed to explain SSB induction by MNNG (23), but as stated earlier, a portion of the SSB seen by alkaline elution appeared to be alkaline-labile sites. A main advantage of assaying MNNG-induced SSB by the technique used in this report is the increased sensitivity, compared to that used in previous work (23, 30).

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