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PROTECTIVE EFFECTS OF HISTONES AGAINST DRUG-INDUCED ALTERATIONS OF DEOXYRIBONUCLEIC ACID IN THYMUS CHROMATIN

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Abstract—When *S*-(1, 2-dichlorovinyl)-L-cysteine is cleaved by a lyase *in vitro*, a very reactive alkylating fragment (AF) is produced which can combine with a variety of acceptors, including DNA. Using ^{35}S as tracer of AF, it was shown that histone, added as such, and particularly in the form of bovine thymus chromatin reacted with AF and greatly decreased the extent to which AF combined with DNA. In contrast to DNA treated directly with AF, DNA which was isolated from AF-treated chromatin retained most of its primer activity for DNA and RNA polymerases and its susceptibility to hydrolysis by pancreatic DNase I. These results illustrate the role which histones and perhaps other nuclear proteins may have in protecting DNA against interaction with various toxic agents. They may also have a bearing on the susceptibility of various cells, tissues and species to the effects of proximate carcinogens and other proximate toxicants formed by metabolic reactions of toxic or therapeutic agents *in vivo*.

APLASTIC ANEMIA can be observed in a calf 3–4 weeks after administration of a single, small dose of *S*-(1, 2-dichlorovinyl)-L-cysteine (DCVC).[1] We reported previously that DNA isolated from bone marrow, lymph nodes and thymus glands of calves about 2 weeks or later after their treatment with DCVC had abnormal physical properties.[2] These specimens contained a component with a markedly lower buoyant density, and they were more resistant to thermal denaturation, with high T_m values and, under certain conditions, biphasic melting profiles. These changes in physical properties of DNA occurred coincident with its complete loss of template activity for DNA[3] and RNA[4] synthesis when tested with bacterial polymerases. In contrast, the DNA isolated from liver, kidney, spleen and intestinal mucosa of the same animals was apparently normal, judged by the same criteria.

Upon cleavage with a lyase from bovine kidneys, DCVC yields a fragment which has some properties of an alkylating agent.[5] Using this enzyme *in vitro*, it has been shown[6] that DNA isolated from a number of sources reacts with the generated alkylating fragment (AF)* and that the resultant product has similarly altered physical and biological properties. Synthetic homo- and heteropolymers of nucleotides can also serve as acceptors of AF.[7] Since AF attacks DNA of hemopoietic tissues in intact animals, apparently preferentially, some kind of protection appears to be afforded to the DNA of other tissues. Nuclear DNA in chromatin is complexed with

* AF, an alkylating fragment which contains the thiovinyl group of DCVC; produced by the action of lyase on DCVC.

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histones and with other proteins.[8] Replication and transcription of DNA are preceded by temporary, localized separation from proteins and unwinding of a double strand.[9] Modification of DNA by AF may occur coincident with these processes in rapidly proliferating tissues more readily than in others. A comparison of the interactions of AF with free and chromatin-bound DNA is, therefore, pertinent.

EXPERIMENTAL

Materials. Calf thymus DNA was isolated according to the method indicated earlier.[2] Chromatin was isolated from calf thymus nuclei and purified following the procedure of Marushige and Bonner.[10] The products had an average mass ratio of DNA 1·0, histone 1·15, RNA 0·020 and non-histone protein 0·54. Lyophilized calf thymus whole histone was purchased from Worthington Biochemicals Corp., Freehold, N.J. Concentrates of bovine kidney DCVC lyase were prepared as previously described.[5] [^{35}S]DCVC was synthesized in this laboratory[1] from [^{35}S]L-cystine (Schwarz Bio Research). *Escherichia coli* RNA polymerase, EC 2.7.7.6 (sp. act. 354 units/mg of protein), was purchased from Miles Laboratories, Inc., Kankakee, Ill., and *E. coli* DNA polymerase, EC 2.7.7.7 (sp. act. 5000 units/mg of protein), was from Biopolymers, Chargin Falls, Ohio. Bovine pancreatic crystalline deoxyribonuclease, EC 3.1.4.5 (DNase I) was obtained from Worthington Biochemicals corp., Freehold, N.J. Nucleoside triphosphates were obtained from Schwarz BioReasearch, Orangeburg, N.Y. and from P. L. Laboratories, Milwaukee, Wis. Hyamine hydroxide, 1 M in methanol was purchased from Packard Instrument Co., Downer Grove, Ill. All other chemicals were of reagent quality. Deionized, distilled water was used throughout.

Analyses. DNA and RNA polymerase were assayed as described previously.[3,4] DNase I activity was measured in Tris–HCl, pH 7·0, containing $MgCl_2$ according to Kunitz.[11] DCVC-lyase was determined by measuring pyruvate formed.[12] Analytical methods for DNA, RNA and protein have been cited earlier.[2] Histone was assayed by the method of Lowry *et al.*[13] as modified by Eggstein and Kreutz[14] using calf thymus whole histone as standard.

Reaction of chromatin with [^{35}S]*DCVC and lyase.* To generate [^{35}S]AF, a solution of [^{35}S]DCVC (approx. 1×10^5 cpm) and lyase were used, as described in the various experiments. Chromatin, DNA or DNA plus histone, in 0·1 M Tris–HCl, pH 7·5, were added to this solution and the mixtures (1 ml) were kept at 37° for various periods. Changes in different parameters are described under each figure. After incubation, the reaction mixture was quickly cooled in ice and fractionated into DNA, histone and lyase as described below.

Fractionation of chromatin–lyase mixture. Each reaction mixture was treated with 4 vol. of ice-cold absolute ethanol, stirred and kept at 0° for 15 min. It was then centrifuged at 10,000 *g* for 20 min and the supernatant was discarded. The precipitate was dispersed and washed twice in 5 ml of ice-cold absolute ethanol. Control experiments showed that after two alcohol washings, unreacted, labeled DCVC was completely removed. The precipitate was then dispersed in 2 ml of 0·2 N HCl and stirred for 30 min at 2°. It was then centrifuged at 16,000 *g* for 20 min and the precipitate was re-extracted with another 2-ml portion of 0·2 N HCl. These combined extracts contained histone.

The precipitate was then dispersed and extracted twice with 2 ml of 0·1 M Tris–HCl, pH 8·0, and stirred for 3 hr at 4° to dissolve DNA. It was centrifuged at 16,000

g for 20 min. The precipitate consisted of denatured lyase which was dissolved by heating at 60° in 4 ml hyamine hydroxide. When chromatin was present in the reaction mixture, the Tris–HCl extract also contained non-histone protein bound to DNA. To separate this protein, 1 ml of 50% trichloroacetic acid (TCA) was added to the Tris–HCl extract (4 ml), and the mixture was heated in a 95° water bath for 15 min.* It was centrifuged at 10,000 *g* for 15 min and the precipitate was washed twice with 1-ml portions of 10% TCA. The combined supernatants contained hydrolyzed DNA while the precipitate was non-histone protein which was then dissolved in 2 ml hyamine hydroxide. Aliquots of each fraction were used to determine radioactivity in a liquid scintillation spectrometer as previously described.[6] Control experiments indicated quantitative recovery of each component following these procedures.

Pretreatment of DNA and chromatin with AF. A solution (1 ml) containing 2·5 μmoles DNA-P (as DNA or chromatin), 10 μmoles DCVC, 100 μmoles Tris–HCl, pH 7·5 and 1·2 mg lyase (6·4 units) was incubated at 37°. After 30 min, the solution was cooled in ice and dialyzed at 4° exhaustively against 0·1 M Tris–HCl, pH 7·5. In some cases, the proteins from chromatin were precipitated with a chloroform–isoamyl alcohol mixture (24 : 1, v/v) and the soluble DNA was isolated by precipitation with ethanol and purified by reprecipitation. The DNA thus recovered from chromatin contained about 1 per cent protein and had normal melting profile and absorption spectrum.

Activation of DNA and chromatin. For the DNA polymerase assay, normal or AF-treated DNA or chromatin was activated by heating a solution in a boiling water bath for 10 min and cooling rapidly in ice. This treatment of chromatin did not dissociate the protein from DNA. Chromatin had less template activity than free DNA, and since chromatin was less susceptible to hydrolysis by DNase I, the activation by nicking with DNase[15] was not effective. Only heat-denatured specimens were used as template for DNA polymerase assays, while unheated specimens were used for RNA polymerase assays.

RESULTS

Reaction of [^{35}S]AF with chromatin components. When chromatin, histone, DNA or mixtures of these weje treated with [^{35}S]DCVC and different amounts of lyase for 30 min, the TCA-insoluble fraction which consisted of lyase, DNA, histone and chromatin proteins contained radioactivity which could not be removed by washing with TCA. Figure 1 shows that the total incorporation of the [^{35}S]AF into acid-insoluble materials was proportional to lyase activity as measured by pyruvate production. Histone and chromatin did not inhibit the lyase (Fig. 1).

Figure 2 shows a comparison of ^{35}S found in the fractionated components when DNA, chromatin or DNA plus histone were treated with DCVC and lyase. In the presence of histone, the combination of [^{35}S]AF with DNA and with lyase was slower and much less extensive (only about 50 per cent; Fig. 2B) than when DNA and lyase were the only acceptors (Fig. 2A). When the chromatin complex served as the potential acceptor (Fig. 2C), the uptake of ^{35}S by DNA was very slow and small. The histone of chromatin was a much better acceptor than isolated histone mixed

* This may have solubilized some ^{35}S bound to protein.

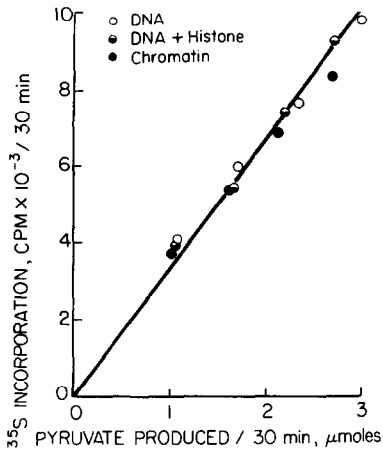

FIG. 1. Proportionality between lyase reaction (pyruvate production) in the presence of DNA, DNA plus histone or chromatin and incorporation of ^{35}S from [^{35}S]DCVC into TCA precipitate. Each reaction mixture contained 0·01 M [^{35}S]DCVC (9·85 × 10^4 cpm), 0·1 M Tris–HCl, pH 7·5, varying amounts of lyase (6·4 units/ml) and 300 μg DNA (as DNA or chromatin). Histone concentration when added was 375 μg/ml.

with DNA (Fig. 2B). All three of these components, as well as the lyase protein, are subject to competitive interaction with AF.

The amout of ^{35}S which became bound to free DNA reached a maximum with a DNA concentration of about 0·3 mg/ml (Fig. 3A), which had little effect on the uptake of ^{35}S by the lyase protein (Fig. 3A and B). In the presence of free histone, the uptake of ^{35}S by DNA was reduced by more than 50 per cent, but it became more dependent on the concentration of DNA (Fig. 3B). When present in the form of chromatin (Fig. 3C), the same amounts of DNA combined with much less ^{35}S than free DNA in the absence (Fig. 3A) or presence (Fig. 3B) of free histone. The histone

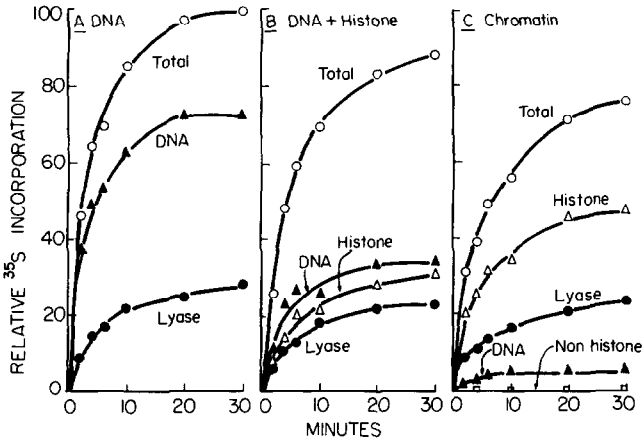

FIG. 2. Incorporation of [^{35}S]AF as a function of time. Reaction mixture containing 0·01 M [^{35}S]DCVC (9·96 × 10^4 cpm), 0·1 M Tris–HCl, pH 7·5, 1·3 mg lyase (3·5 units) and 315 μg DNA (as DNA or chromatin) or 315 μg DNA plus 325 μg histone (as free histone or chromatin) was incubated at 37° for various times. Individual fractions were isolated as described under the Experimental section. Each point is compared to the maximum incorporation taken as 100.

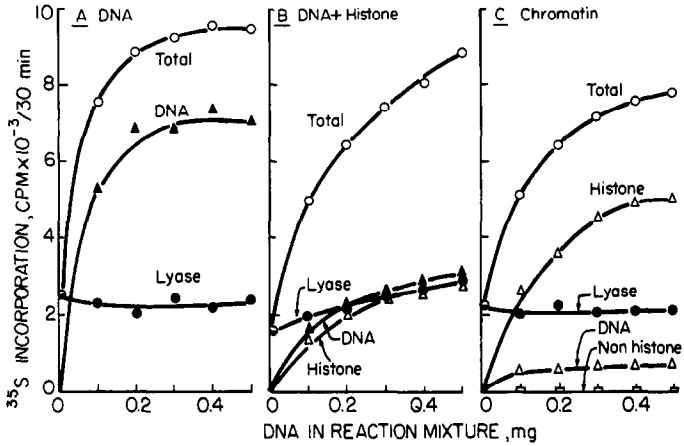

FIG. 3. Incorporation of [^{35}S]AF as a function of DNA concentration in the reaction mixture (present as chromatin or DNA). Each reaction mixture contained 0·01 M [^{35}S]DCVC ($8{\cdot}37 \times 10^4$ cpm), 0·1 M Tris–HCl, pH 7·5, 1·2 mg lyase (3·2 units) and varying amounts of DNA. Chromatin had a DNA : histone mass ratio of 1 : 1·25. Equivalent amounts of histone were present in B and C.

of chromatin was a better acceptor of the ^{35}S fragment than free histone. The isolated non-histone proteins of chromatin did not contain ^{35}S (Fig. 3C).

The amounts of the [^{35}S]AF which combined with DNA, histone or lyase were linearly related to the amount of lyase present (Fig. 4). With all concentrations of lyase, the histone present in the free form (Fig. 4B) and in the form of chromatin (Fig. 4C) afforded much protection for the DNA (compared with Fig. 4A). When the histone fraction of chromatin approached saturation with the [^{35}S]AF, the amounts of AF bound by DNA increased rapidly (Fig. 4C). Similar, concentration-dependent amounts of [^{35}S]AF were bound by the lyase whether free or chromatin-bound histone was present.

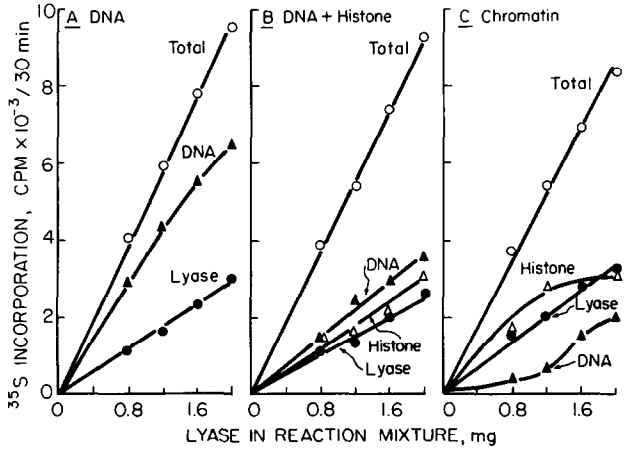

FIG. 4. Incorporation of [^{35}S]AF as a function of lyase activity. Reaction conditions were the same as described in Fig. 2, except that different amounts of lyase were used and DNA concentration was 300 μg and histone was 375 μg/ml.

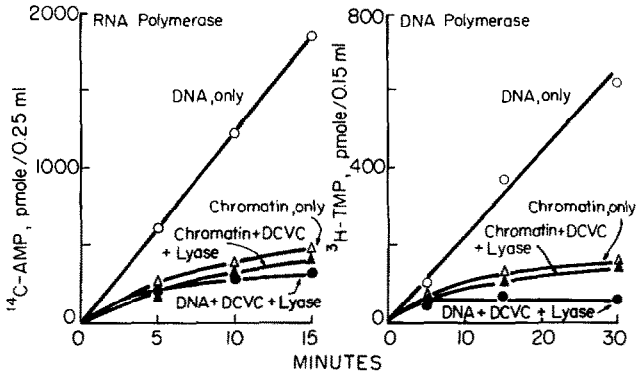

FIG. 5. Effect of DCVC and lyase addition on template activity of chromatin and DNA in RNA and DNA polymerase assay systems. Concentration of primer, 25 nmoles DNA-P; DCVC, 10 μmoles/ml; and lyase, 10 μg.

When histones isolated from thymus glands were treated with [^{35}S] DCVC and lyase and fractionated by means of electrophoresis through polyacrylamide gel, radioactivity was associated with all five histone fractions but not with the small amount of residual other proteins.*

Template activity of AF-treated chromatin and its DNA fraction. Compared to the template activity of normal bovine thymus DNA, that of an equal amount of DNA in the form of chromatin was much less, about 25 per cent for RNA polymerase and 20 per cent for DNA polymerase. As shown in Fig. 5, these template activities of chromatin were slightly decreased when DCVC and lyase were added to the polymerase assay systems. However when the AF was being generated in polymerase assay systems containing free DNA, the polymerase reaction was quickly and greatly inhibited, especially in the DNA polymerase assay.

Pretreatment of chromatin with DCVC and lyase for 30 min, caused slight inhibition of its template activity for both polymerases (about 15–20 per cent inhibition). On the other hand, similar pretreatment of free DNA inhibited its template activity by 90 per cent for RNA polymerase and 95 per cent for DNA polymerase, as shown in Table 1. In contrast, the DNA fraction extracted from chromatin which had been pretreated with AF, when tested in polymerase assays, retained about 90 per cent of the template activity of DNA extracted from untreated chromatin. The template activity of DNA measured with DNA polymerase was lost more rapidly during its reaction with AF (Fig. 6) than that for the RNA polymerase. A slow rate of decline but much less total inhibition was also noticed for the template activity of chromatin and its DNA fraction when the chromatin was treated with AF.

Addition of DCVC alone to the polymerase assay system or pretreatment with lyase alone did not produce any change in the template activity of DNA or chromatin for the two bacterial polymerases.

Hydrolysis of treated chromatin by DNase I. The rate of hydrolysis of chromatin by DNase I was initially much slower, and it also declined more quickly than that of free DNA. Free DNA continued to serve as substrate for this nuclease, and at the end of 60 min 28 per cent of the DNA was hydrolyzed. As shown in Fig. 7, DNA

* Unpublished work by the authors.

TABLE 1. EFFECT OF PRETREATMENT WITH DCVC AND LYASE ON THE TEMPLATE ACTIVITY OF DNA AND CHROMATIN FOR *E. coli* RNA AND DNA POLYMERASES

Template*	RNA synthesis (pmoles [^{14}C]AMP/ 15 min/0·25 ml)	DNA synthesis (pmoles [^{3}H]TMP/ 30 min/0·15 ml)
DNA, calf thymus, untreated	1830	740
Chromatin, untreated	480	115
DNA extracted from untreated chromatin	1850	700
DNA extracted from AF-treated chromatin	1700	620
DNA, AF-treated	200	25
Chromatin, AF-treated	390	85

* See text for method of pretreatment. DNA or chromatin was thermally denatured for use in DNA polymerase assay, but not for RNA polymerase assay.

pretreated with AF was much less susceptible to DNase I attack, and maximum hydrolysis was only about 10 per cent. AF-pretreated chromatin or DNA isolated from such chromatin behaved like their untreated counterparts upon hydrolysis by DNase I.

DISCUSSION

Enzymatic cleavage of DCVC produces pyruvate, ammonia, chloride and a thiovinyl fragment (AF), which has properties of an alkylating agent.[5] AF can combine with several acceptors. Among these are small molecules such as cysteine, glutathione and 4-(*p*-nitrobenzyl)-pyridine,[5] biological polymers like DNA,[6] synthetic polynucleotides[7] and some proteins including DCVC lyase.[5,12] It is likely that such combination of AF with vital cell components like protein and DNA is responsible for the toxic properties of DCVC.

We have shown in the present studies that chromatin can also combine with AF. When this occurs, the DNA portion of the chromatin complex is largely protected from attack by AF, as shown in Figs. 2–4. Although free histone afforded some protection to the DNA, the bound histones in chromatin were more effective, and in this case, most of the AF became firmly bound to histone. When both histone and

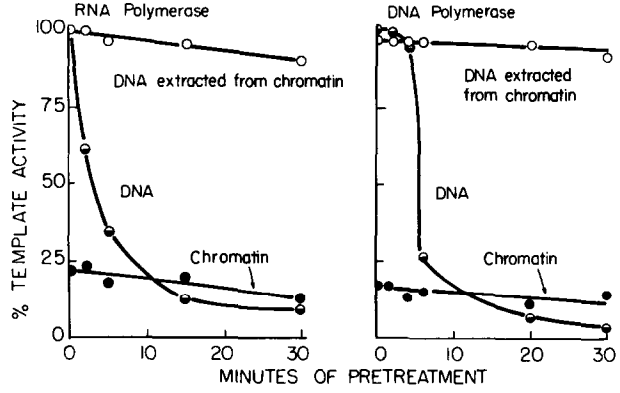

FIG. 6. Effect of pretreatment with AF of DNA and chromatin on their template activity for DNA and RNA polymerases. See Experimental section for method of pretreatment: primer concentration was 25 nmoles DNA-P.

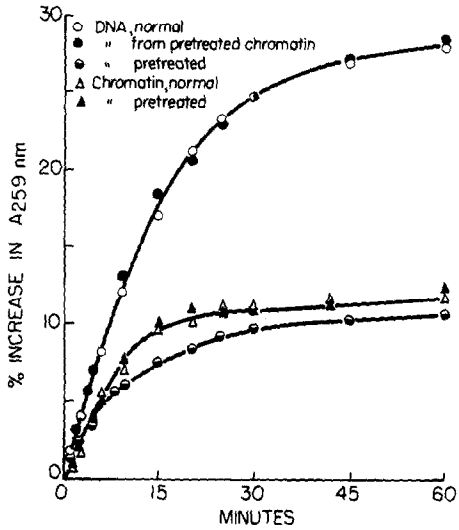

FIG. 7. Hydrolysis by pancreatic DNase I of DNA and chromatin pretreated with DCVC and lyase. Each reaction mixture contained 350 μmoles Tris–HCl, pH 7·0, 100 μg DNA (as DNA or chromatin), 17·5 μmoles Mg^{2+} and 2·5 μg crystalline pancreatic DNase I. Total volume in each case was 3 ml.

DNA were added in the "free" form, however, each competed for AF (Figs. 2–4A and B).

Like normal, free DNA, DNA isolated from chromatin which had been treated with AF served as template for replication and transcription (Table 1). In this respect, it differed from free DNA similarly treated *in vitro*, which lost template activity progressively and had altered physical properties.[6] Even a very small amount of lyase when added with DCVC to RNA or DNA polymerase assay systems inhibited DNA template activity extensively (Fig. 5).

In contrast, under similar conditions, chromatin retained its relatively low template activity almost completely. Lower template activity of chromatin for RNA synthesis has also been reported previously by others.[10,16,17] It has been suggested that this low template activity is due to relatively small regions of exposed DNA in chromatin.[18,19]

Pretreatment of chromatin *in vitro* with AF caused only a slight lowering of its template activity (Table 1). This could be due to the reaction of AF with exposed DNA regions in the chromatin. A small amount of ^{35}S which combined with DNA of [^{35}S]AF-treated chromatin (Fig. 2C) supports this interpretation. The inhibition of template activity of chromatin was gradual during prolonged reaction (Fig. 6), and the over-all rate was very low as compared to free DNA which lost about 90 per cent of the template activity very quickly. During treatment of chromatin with AF, most of the DNA strand remained apparently unreacted, however, so that it retained about 90 per cent of the template activity of DNA isolated from untreated chromatin. Protection of DNA in the chromatin complex is also indicated by the fact that DNA isolated from the complex was as susceptible to hydrolysis by DNase I as normal DNA. Whereas AF-treated DNA was much more resistant to hydrolysis by DNase I, treatment of chromatin with AF did not decrease its susceptibility to such hydrolysis (Fig. 7).

The evidence presented here is in harmony with experiments of an entirely different nature which indicate that about half of the DNA in chromatin from bovine thymus gland is physically covered by protein.[20] In chromatin from rat thymus glands, even a larger proportion may be covered.[21] The protein inhibited, in part at least, the alkylation of DNA in nucleoproteins from *E. coli* by nitrogen mustard,[22] or of DNA in murine hematopoietic stem cells by various alkylating agents.[23] Histones protected DNA in chromatin against its combination with an acridine dye (proflavin)[24] and against hydrolysis by DNase.[25] To what extent DNA in various cells and in different developmental stages would be similarly protected is uncertain.

The present observations concerning protection of DNA by histone against attack by the enzymatically released thiovinyl fragment of DCVC may account for the remarkable specificity in the appearance of abnormal DNA in some organs of the calf after administration of DCVC. Two weeks after a single injection (3 mg/kg body weight) of DCVC into a calf, DNA isolated from hemopoietic tissues (lymph nodes, bone marrow and thymus) was found to have altered physical and biological properties.[2-4] DNA from other tissues, however, had normal properties as measured by the same methods. The cells of target hemopoietic organs replicate rapidly; DNA is constantly being synthesized much more rapidly than DNA in liver and many other tissues. Although not completely understood, it is generally assumed that histone controls the replication process of DNA in the cell.[19] Therefore, it may be assumed that during replication of DNA, histone separates completely or partially from DNA, allowing specific regions to be duplicated. The AF from DCVC reacts perhaps with free DNA or free DNA regions, while such replication processes occur in the cell. The DNA so altered becomes incapable to serve as template for DNA and RNA polymerase.

The reaction of chromatin and various histones with AF necessitates further study of the nature of such binding. Such work is in progress now.

It is likely that the protective effect of histones and other proteins in the chromatin complex is only one of several factors which govern the susceptibility of DNA in various tissues and species to toxic agents. Among other such factors are the distribution of enzymes which can bring about metabolic changes in the administered compounds to convert them into ultimate toxicants, or to inactivate them. Also, the rates of DNA replication and the presence of enzymes which can bring about repair of damaged DNA must be taken into account.

While our observations can serve to explain the effects of DCVC observed in the bovine animal, we are cognizant of several phenomena which are not accounted for. Of various tissues examined, the bovine liver and kidney are among the best sources of DCVC lyase activity.[5,12] Further study of its distribution and of factors affecting its activity in organs of both susceptible[1] and resistant[26,27] species are clearly indicated. If the reactive alkylating fragment were produced mainly in liver and kidney, how is it transported to the hemopoietic centers without losing its activity? It is reasonable to suspect that, coincident with its replication, the DNA in the chromatin complexes of all tissues and species becomes temporarily accessible to alkylation or other chemical modifications. Is the protective effect of histone greater in those species in which DCVC does not elicit blood dyscrasia?[26,27] Finally, we found[3] that DNA isolated from the mucosa of the small intestine, in contrast to that from the hemopoietic tissues of calves which are so severely affected by DCVC, had normal proper-

ties. The turnover of cells in the intestinal mucosa is very rapid[28] with a high mitotic rate and a rapid replacement time for DNA.[29] It is quite possible that any abnormal DNA which had formed in such cells after administration of DCVC had been replaced by normal DNA by the time our animals were slaughtered in the earlier studies.[2–4] It is also possible that histone complexed with DNA in various tissues cannot provide the same protective effects. Animals or tissues treated with DCVC would appear to offer a valuable model for the study of differences of resistance to toxic agents by various species and tissues.

While this study has focused on the histone fraction of the chromatin complex, the non-histone chromosomal proteins may also be involved in the response to or protection against toxic agents by specific tissues. Allfrey *et al.*[30] have recently emphasized the importance of acidic proteins of the mammalian cell nuclei and their metabolic changes for the control of template activity of DNA for RNA polymerase.

Singer and Fraenkel-Conrat[31] have discussed the strong protecting action of the tobacco mosaic virus protein against the reaction of its associated RNA with some mutagenic agents. They consider protein-encapsulated double-stranded DNA of T_2 or T_4 phages similarly resistant as free double-stranded DNA to modification by mutagenic agents in the hydrogen-bonded regions of the bases.

Histone and other proteins associated with DNA may have a general protective effect on DNA against toxic compounds with which they can react. Such effects may be important in governing the susceptibility of various cells, tissues and species to the effects of proximate carcinogens[32] and proximate toxicants,[3] in general, which are formed by metabolic reactions *in vivo*. In this light, studies designed to determine the extent to which DNA of chromatin is "unprotected by proteins"[20,21] assume special significance even though their results and interpretations have not been concordant to date.

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