Reactivity of Thymine to γ Rays in HeLa Chromatin and Nucleoprotein Preparations†

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ABSTRACT: The radiation chemical reactivity of the thymine residues in the DNA of four nucleoprotein preparations from HeLa S3 cells was investigated: (1) native chromatin, (2) reconstituted chromatin, (3) reconstituted DNA–histone complexes, (4) reconstituted DNA–non-histone chromosomal protein complexes. The γ-ray-induced formation of tritiated water from [*Me*-^{3}H]thymine was measured to assess the reactivity of the thymine methyl group. The formation of ring-saturated radiation products of the 5-hydroxy-6-hydroperoxydihydrothymine type was determined by selective degradation with alkali and acid. Linear dose–response curves were found in the low-dose region. It was found that the chromosomal proteins effectively protected the thymine residues from indirect radiation action, *i.e.*, attack by radicals formed by water radiolysis, relative to free DNA. The relative reactivities for both reactions of thymine were: free DNA $\gg$ native chromatin = reconstituted chromatin > reconstituted DNA–histone complexes > reconstituted DNA–non-histone chromosomal protein complexes. The observation that native and reconstituted chromatin was radiation chemically identical lends support to the notion that such preparations have similar or identical structural features. According to our results it appears unlikely that chromatin contains substantial portions of naked DNA. It is particularly interesting to note in support of recent chromatin models that the thymine residues were more reactive in chromatin than in reconstituted DNA–histone or DNA–non-histone chromosomal protein complexes despite the fact that chromatin contained about twice the amount of protein.

The DNA *in situ* in a mammalian cell is strongly protected from the effects of ionizing radiation as compared to free DNA (see, *e.g.*, Okada, 1970). The efficiency of the formation of thymine damage by γ rays was found to be several magnitudes lower in Chinese hamster ovary cells than in free DNA irradiated in aerated nonprotective medium (Roti Roti and Cerutti, 1974). Damage to DNA by γ rays in aqueous solutions *in vitro* or inside the cell is mostly due to the attack by radicals produced by water radiolysis, most importantly OH radicals (indirect radiation action; Johansen and Howard-Flanders, 1965; Roots and Okada, 1972; Roti Roti and Cerutti, 1974). The efficiency of damage production in DNA therefore depends on the effective concentration of such radicals at the reaction sites and is expected to be strongly influenced by the presence of radical scavengers in the proximity of the DNA. Hydration of the DNA and accessibility of the reaction sites to radicals are further important factors. In eukaryotic chromatin the DNA is associated with a large number of histones and non-histone proteins (Bonner *et al.*, 1968). The chemical composition of the chromosomal proteins together with the mode and tightness of their binding to the DNA in chromatin are expected to determine the effectiveness with which they shield the DNA from indirect radiation action. Studies of the radiation chemical reactivity of the DNA in chromatin may, therefore, besides their importance to radiobiology, yield information concerning the manner in which chromosomal proteins are associated and interact with DNA.

In this paper we compare the reactivity of the thymine residues to γ rays in isolated native HeLa S3 cell chromatin, reconstituted chromatin, and reconstituted nucleoprotein complexes. The reconstituted nucleoprotein complexes consist of DNA and histones or DNA and non-histone chromosomal proteins. The reactivity of the thymine methyl group was studied by measuring the formation of [^{3}H]H_2O from [*Me*-^{3}H]thymine which follows attack by OH radicals (Swinehart *et al.*, 1974) and the formation of products of the 5-hydroxy-6-hydroperoxydihydrothymine type (t′) involving attack by radicals at the 5,6-double bond of the ring. A new procedure developed by Hariharan and Cerutti was used for the determination of t′ and is described under Materials and Methods. The thymine methyl group in double stranded DNA is located on the helix periphery while the thymine ring is intercalated toward the helix center. Both the methyl substituent and the 5,6-double bond of thymine face the major groove of the DNA helix. The reactions studied here are, therefore, expected to be most sensitive to changes in chromatin structure affecting protein binding to the major groove.

Materials and Methods

Cell Maintenance and Labeling. HeLa cells were maintained in suspension culture with Joklik-modified Eagle's minimal essential spinner-medium supplemented with 3.5% each of calf serum and fetal calf serum.

Cells were subcultured into roller bottles containing 150 ml of basal minimal essential medium (BME) supplemented with 3.5% each of calf serum and fetal calf serum at 5–7.5 $\times$ 10^7 cells/bottle and growth was continued for 2–3 days. Twenty hours before the cells were to be harvested the culture medium was replaced by 50 ml of medium (BME) containing [^{3}H]-thymidine (10 μCi/ml, specific activity 50–60 Ci/mmol).

Isolation of Nuclei and Chromatin. Labeled cells were washed twice with isotonic saline and then harvested from the glass surface by trypsinization. All subsequent procedures were carried out at 4°. Nuclei were isolated by the method of Stein and Borun (1972) and chromatin was prepared as described

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by Stein and Farber (1972). Cells were washed in isotonic saline and twice in calcium free Earle's balanced salt solution, and lysed in 60 volumes of 80 mM NaCl–20 mM EDTA–1% Triton X100 (pH 7.2), and nuclei were pelleted by centrifugation at 1000*g* for 10 min. The nulcei were washed twice with lysing solution and twice with 0.15 M NaCl–0.01 M Tris (pH 8.0). Nuclei isolated utilizing this procedure are free of visible cytoplasmic contamination when examined by phase-contrast microscopy. Chromatin was prepared by lysing the nuclei in water, pelleted by centrifugation at 1200*g*, and then resuspended in 0.001 M sodium phosphate (pH 7.0).

Preparation of Reconstituted Chromatin. A. ISOLATION OF DNA. DNA was prepared from exponentially growing HeLa S3 cells by the method of Marmur (1963), treated with ribonuclease and Pronase and extracted with phenol prior to use. DNA size was measured by alkaline sucrose gradient sedimentation and found to be similar to that in native chromatin.

B. ISOLATION OF CHROMOSOMAL PROTEINS. All procedures were carried out at 4°. Chromatin was dissociated in 3 M NaCl–5 M urea–0.01 M Tris (pH 8.3) and DNA was pelleted by centrifugation at 100,000*g* for 48 hr. The supernatant containing the chromosomal proteins was fractionated into histone and non-histone chromosomal proteins by the QAE-Sephadex chromatographic method of Gilmour and Paul (1970) as previously described by Stein *et al.* (1972).

C. RECONSTITUTION OF CHROMATIN AND NUCLEOPROTEIN COMPLEXES. All procedures were carried out at 4°. Chromatin was reconstituted as previously described by Stein and Farber (1972). DNA, non-histone chromosomal proteins and histones were combined in 3 M NaCl–5 M urea–0.001 M sodium phosphate (pH 8.3) at a DNA:histone:non-histone chromosomal protein ratio of 1:2:2 w/w. This was followed by gradient dialysis against 5 M urea–0.001 M sodium phosphate (pH 8.3), containing successively decreasing amounts of NaCl. After removal of NaCl the reconstituted chromatin was pelleted by centrifugation at 20,000*g* for 30 min, resuspended in 0.001 M sodium phosphate (pH 8.3), repelleted by centrifugation at 20,000*g* for 30 min, and resuspended in 0.001 M sodium phosphate (pH 7). Nucleohistone complexes and DNA–nonhistone chromosomal protein complexes were prepared in a similar manner.

Nucleic Acid and Protein Determinations. The DNA and protein contents of chromatin and nucleoprotein complexes were determined as follows. Histones were extracted with 0.4 N H_2SO_4 at 4°; nucleic acids were extracted with 1 N perchloric acid at 90° and the remaining non-histone chromosomal proteins were solubilized in 1 N sodium hydroxide. The amount of proteins in the histone and nonhistone chromosomal protein fractions was assayed by the methods of Lowry *et al.* (1951) and the amount of DNA nucleic acid fraction was assayed by Burton's (1965) modification of the diphenylamine reaction.

Irradiation Conditions. Irradiation was carried out with a Mark I ^{137}Cs source, Model 35, from J. L. Shepherd and Associates, Glendale, Calif. The dose rate was 9.1 krads/min. All irradiation was carried out under conditions of aeration at 0° (ice–water bath). One-milliliter nucleoprotein suspensions (OD_{260} 2.70) in 0.001 M sodium phosphate (pH 7.0) were irradiated in test tubes precleaned according to Van der Schans *et al.* (1973).

Assays for γ-Ray-Induced Thymine Damage. A. FORMATION OF [^{3}H]H_2O FROM [*Me*-^{3}H]THYMINE. Immediately following irradiation cold CCl_3COOH was added to a final concentration of 5% and after 45 min at 4° the resulting precipitate was spun down. The acid-soluble extracts were neutralized and analyzed for their [^{3}H]H_2O content by ion-exchange chromatography according to Swinehart *et al.* (1974) as described below. The samples were placed on an ion-exchange column of diameter 1.1 cm, containing a top layer of 4 ml of Bio-Rad AG1-X10 (OH^-) and separated by a layer of sand 4 ml of Bio-Rad AG50-X8 (H^+) on the bottom. The columns were eluted with decarbonated water and 1-ml fractions were collected. One major radioactivity peak was obtained in fraction 7–10 consisting of [^{3}H]H_2O, *i.e.*, at an elution volume identical with authentic [^{3}H]H_2O. No radioactivity was eluted in these fractions if the samples were evaporated to dryness and the content was taken up in a small volume of solvent before application on the ion-exchange columns. The acid precipitate was analyzed for products of the 5-hydroxy-6-hydroperoxydihydrothymine type (t′) according to Hariharan and Cerutti (submitted for publication) and is described below under B.

For an estimate of the total amount of water produced by the reaction of the thymine methyl group with OH radicals the values for the formation of [^{3}H]H_2O have to be multiplied by an approximate factor of 5 to account for the primary isotope effect and the probability of the abstraction of tritium instead of hydrogen. Since the [*Me*-^{3}H]thymine was prepared by catalytic hydrogenation of 5-dichloromethyluracil with tritium gas the methyl substituent contains maximally two tritium atoms. At the specific activity of 54 Ci/mmol used in our experiments the labeled thymine methyl group contains on the average 1.7 tritium atoms. The primary isotope effect is estimated at 3 (Pryor, 1966). The extrapolation factor therefore is $1.7 \times 3 \simeq 5$.

B. DETERMINATION OF PRODUCTS OF THE 5-HYDROXY-6-HYDROPEROXYDIHYDROTHYMINE TYPE (t′). The principle of the assay is similar to the reductive assay for thymine radiation products of Hariharan and Cerutti (1971, 1972) but the new procedure proved to be simpler and more sensitive. Furthermore, the values for t′ obtained by the present method can more readily be extrapolated to total thymine ring destruction since the assay is carried out under conditions in which the polymer is completely denatured. A complete discussion of the base–acid degradation assay will be given elsewhere. The acid precipitates obtained from the treatment of the irradiated nucleoprotein preparations with CCl_3COOH were dissolved in 1 ml of 0.2 N KOH. The acid precipitable ring saturated thymine products are, therefore, measured by the present procedure and products which are spontaneously released from the polymer are not determined. After 1.5 hr at room temperature 0.33 ml of 2 N HCl was added and the incubation was continued at 70° for 15 min. The samples were then neutralized with KOH and analyzed for their content of acetol by ion-exchange chromatography. Acetol is formed from products of the 5,6-dihydroxydihydrothymine type by the base–acid treatment as has been shown by Burton and Riley (1966). Other radioactive fragments containing the thymine methyl group may also be formed in smaller amounts. The ion-exchange columns contained 0.8 ml of DEAE-Sephadex A25 on top and separated by a layer of sand 7 ml of Bio-Rad AG1-X10 (OH^-) in the middle and separated by another layer of sand, 6 ml of Dowex 50W-X8 (H^+) on the bottom. The columns were eluted with decarbonated water and 1-ml fractions were collected. One major radioactivity peak was obtained in fractions 11–14. The yield of the radioactive fragment produced per ring-saturated product of thymine t′ was determined to be 20% by using poly[d(A-T)]–[*Me*-^{3}H]thymine which was modified with OsO_4 as a model system. OsO_4 selectively introduces 5,6-dihydroxydihydro-

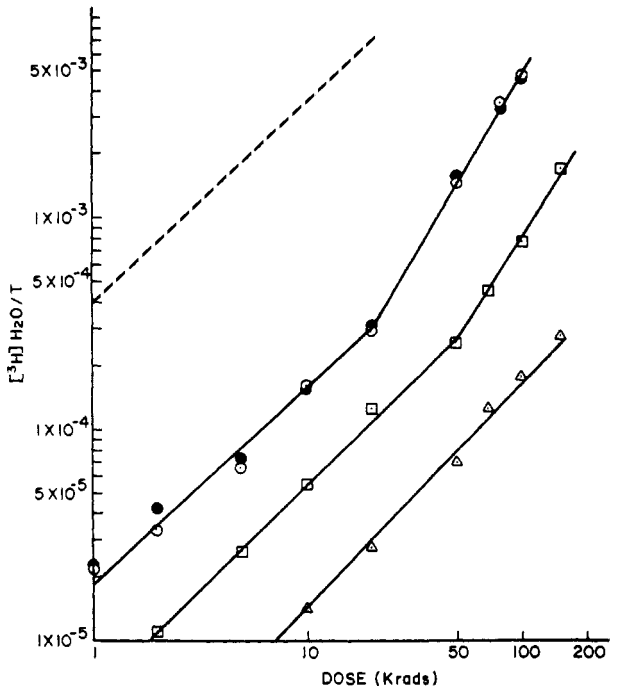

FIGURE 1: Formation of $[^3H]H_2O$ from [*Me*-^{3}H]thymine in nucleoprotein preparations from HeLa S3 cells upon irradiation with ^{137}Cs γ rays in aerated 0.001 M sodium phosphate (pH 7.0): (●—●) native chromatin; (○—○) reconstituted chromatin; (□—□) reconstituted DNA–histone complexes; (△—△) reconstituted DNA–non-histone chromosomal protein complexes; (- - -) free *E. coli* DNA included for comparison from Swinehart *et al.* (1974). The ratio of the amount of radioactivity released in the form of $[^3H]H_2O$ over the total amount of radioactivity originally present in the sample is plotted as a function of dose.

thymine into poly[d(A-T)] or DNA (Burton and Riley, 1966; Beer *et al.*, 1966). Total thymine ring destruction in modified poly[d(A-T)] was determined by measuring the loss in absorbance at 267 nm in 0.2 N KOH.

Results

The reactivity of thymine toward γ rays was compared in four nucleoprotein preparations from HeLa S3 cells: (1) native chromatin, (2) reconstituted chromatin, (3) DNA–histone complexes, and (4) DNA–non-histone chromosomal protein complexes. Isolated chromatin has been shown to retain the transcriptional integrity of the intact cell genome (Bekhor *et al.*, 1969; Paul and Gilmour, 1968; Huang and Huang, 1969). For the preparation of reconstituted chromatin, procedures were used which according to several lines of evidence suggest fidelity of reconstitution (Kleinman and Huang, 1972). Specifically the composition, transcriptional capacity (Stein *et al.*, 1972; Stein and Farber, 1972), binding affinity of histone polypeptides (Stein *et al.*, 1974), and availability of the minor groove of the DNA helix for association with reporter molecules (Gabbay and Stein, unpublished results) are indistinguishable in native HeLa S3 cell chromatin and HeLa S3 cell chromatin reconstituted as described in Materials and Methods. The DNA–histone complexes and the DNA–non-histone chromosomal protein complexes were prepared by the same procedure utilized for reconstitution of complete chromatin. The amounts of histone and non-histone chromosomal protein present in these partially reconstituted nucleoprotein preparations were similar to those found in complete native and reconstituted chromatin. The histone: DNA ratio was 1.10 on a weight per weight basis and the non-histone chromosomal protein: DNA ratio was 0.89. The formation of $[^3H]H_2O$ from [*Me*-^{3}H]thymine and of ring-saturated products of the 5-hydroxy-6-hydroperoxydithydrothymine type (t′) upon irradiation with ^{186}Cs γ rays was determined as described under Materials and Methods using suspensions of the four nucleoprotein preparations in aerated 10^{-3} M phosphate buffer (pH 7) at 0°. The low-salt medium in the absence of divalent metal ions was chosen in order to emphasize primary nucleic acid–protein interaction over effects due to changes in the superstructure of the nucleoprotein. The dose range was 1–150 krad.

Reactivity of the Thymine Methyl Group. As shown in Figure 1, the slopes of the dose–response curves on the log–log scale are close to one indicating approximate linearity of the formation of $[^3H]H_2O$ up to a dose where the value for $[^3H]H_2O$: T, *i.e.*, the ratio of counts released as tritiated water over the total amount of radioactivity originally present in the sample, had reached about 3×10^{-4}. Using an extrapolation factor of 5 (see Materials and Methods) it is calculated that approximately 0.15% of all thymine methyl groups had reacted. At higher doses the slope of the functions is increased. In the case of the reconstituted DNA–non-histone chromosomal protein complexes which show the lowest reactivity a yield of 3×10^{-4} is only reached at the highest dose of 150 krad used in our experiments and no break in the curve is observed. The efficiencies of $[^3H]H_2O$ formed per krad in 10^6-dalton DNA were calculated from the low-dose portion of semilog plots of the dose–response curves and are listed in Table I. The thymine methyl group reacted with highest

TABLE I: Reactivity of Thymine to ^{137}Cs γ Rays in Nucleoprotein Preparations from HeLa Cells.[a]

	Protein: DNA[b]	$[^3H]H_2O$ krad, 10^6 daltons	Rel Efficiency[c]	t′ krad, 10^6 daltons	Rel Efficiency
Native chromatin	1.94	1.42×10^{-2}	1	3.90×10^{-2}	1
Reconstituted chromatin	1.92	1.42×10^{-2}	1	3.90×10^{-2}	1
Reconstituted DNA–histone complexes	1.10	4.90×10^{-2}	2.9	1.35×10^{-2}	2.9
Reconstituted DNA–non-histone chromosomal proteins complexes	0.89	1.54×10^{-3}	9.2	5.92×10^{-3}	6.6
E. coli DNA		0.34[d]		0.23	

[a] Irradiation in aerated 0.001 M sodium phosphate (pH 7) with ^{137}Cs γ rays. The efficiencies of product formation were derived from the slopes of the linear portions of semilog plots of the dose–response curves. [b] The protein to DNA ratios are on a weight per weight basis. [c] *I.e.*, the ratio of the efficiency for native (or totally reconstituted) chromatin over the efficiencies of the less reactive preparations. [d] From Swinehart *et al.* (1974).

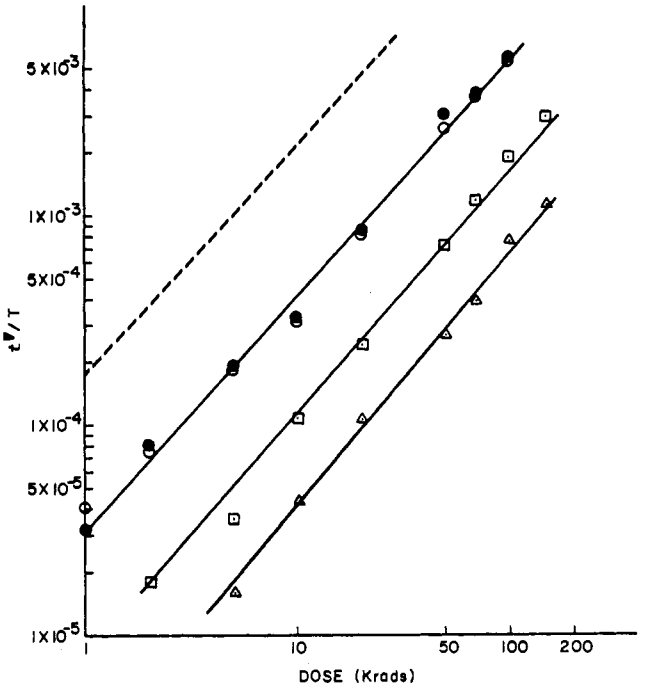

FIGURE 2: Formation of products of the 5-hydroxy-6-hydroperoxydihydrothymine type (t′) in nucleoprotein preparations from HeLa S3 cells upon irradiation with ^{137}Cs γ rays in aerated 0.001 M sodium phosphate (pH 7.0). The products were determined in the acid-precipitable material: (●—●) native chromatin; (○—○) reconstituted chromatin; (□—□) reconstituted DNA–histone complexes; (△—△) reconstituted DNA–non-histone chromosomal protein complex; (- - -) free *E. coli* DNA included for comparison from Swinehart *et al.* (1974). The ratio of the amount of t′ formed over the total amount of radioactivity originally present in the sample is plotted as function of dose.

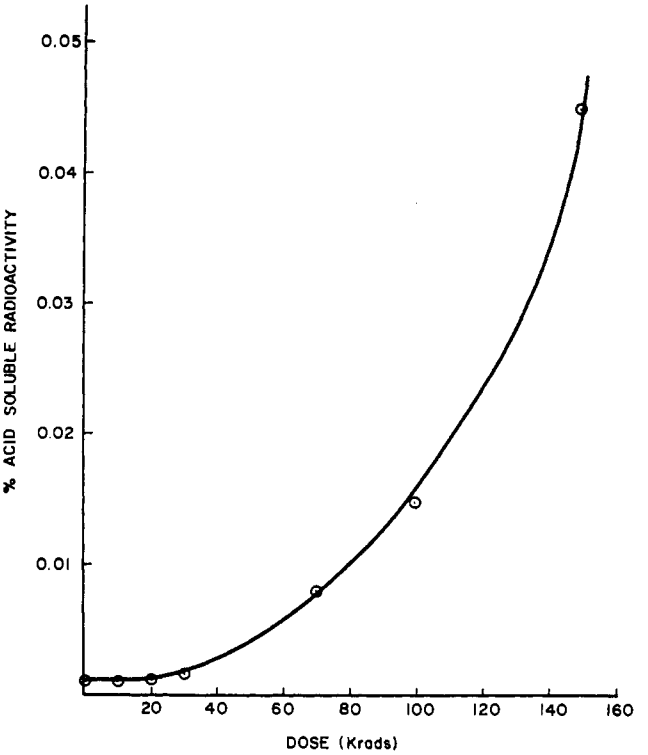

FIGURE 3: Formation of acid-soluble nucleic acid material from native HeLa S3 chromatin upon irradiation with ^{137}Cs γ rays in aerated 0.001 M sodium phosphate (pH 7). The 5% trichloroacetic acid soluble material was determined as a function of dose according to standard procedures.

efficiency, 1.42×10^{-2} in native and totally reconstituted chromatin. An intermediate efficiency of 4.90×10^{-3} was found for nucleohistone complexes and the lowest efficiency, 1.54×10^{-3}, was found for reconstituted DNA–non-histone chromosomal protein complexes. Also listed in the table are relative efficiencies of $[^3H]H_2O$ formation for the different preparations, *i.e.*, the ratio of the efficiency for native (or totally reconstituted) chromatin over the efficiencies of the less reactive preparations. It follows, *e.g.*, that a thymine methyl group in a reconstituted DNA–non-histone chromosomal protein complex is on the average 9.2 times less reactive than in native chromatin which contains more than twice the amount of protein per nucleotide residue. For comparison, values for free *Escherichia coli* DNA are included in the table.

Formation of Ring-Saturated Products of the 5-Hydroxy-6-hydroperoxydihydrothymine Type (t′). Figure 2 gives the dose–response curves for the formation of acid precipitable t′. It should be noted that a fraction of ring-damaged thymine residues are spontaneously released from the polymer and are therefore not measured in our experiments. The curves were essentially linear over the entire dose range from 1 to 150 krads. The efficiencies of product formation calculated from the slopes of the low-dose portions of the curves of semilog plots (not shown) were identical for native and totally reconstituted chromatin at 3.90×10^{-2} t′/krad, 10^6-dalton DNA, 2.9 times lower for nucleohistone at 1.35×10^{-2}, and 6.6 times lower for reconstituted DNA–non-histone chromosomal protein complexes at 5.92×10^{-3}. The data are summarized in the table.

In a series of experiments the question studied was whether radiation-induced DNA degradation could be responsible for the increase in the efficiency of $[^3H]H_2O$ formation above a certain effective dose. On the basis of results obtained with mononucleotide mixtures and *E. coli* DNA (Swinehart *et al.*, 1974) it was expected that the acid-soluble nucleic acid moieties which are released from the nucleoprotein are radiation chemically considerably more reactive than residues in the nucleoprotein complex. Radiation-induced solubilization of DNA, *i.e.*, formation of 5% CCl_3COOH-soluble radioactive material, was, therefore, investigated and is shown in Figure 3 for native chromatin. It is evident from these results that efficient solubilization commences in the same dose range where the change to increased efficiencies for $[^3H]H_2O$ formation is observed.

Discussion

It is evident from our data that both the reaction of the thymine methyl group measured by the formation of $[^3H]H_2O$ and the formation of ring-saturated products t′ are strongly suppressed in all nucleoprotein preparations studied here relative to free DNA in solution. The chromosomal proteins, therefore, effectively shield the DNA from radical attack. The dashed lines in Figures 1 and 2 represent the dose–response curves for free *E. coli* DNA irradiated under non-protective conditions and were included for comparison. It is interesting that the two reactions are suppressed to a different degree, *e.g.*, for native chromatin the efficiency of $[^3H]H_2O$ formation is 24 times lower, the efficiency of t′ formation, however, only 6 times lower than in free DNA. This result is not surprising. Since the reaction at the thymine methyl group occurs at the periphery of the major groove of the DNA helix, it would be expected to be particularly sensitive to the addition of chromosomal proteins. According to most current models for chromatin histone binding occurs preferentially at the major groove of the helix (see Huberman, 1973).

Thymine residues in native chromatin *in vitro* are substantially more reactive toward γ rays relative to residues in the living cell. The efficiency of $[^3H]H_2O$ formation in human embryonic lung fibroblasts WI-38 was 0.32×10^{-3}/krad, 10^6 daltons, *i.e.*, 44 times lower than for HeLa chromatin, that of t′ formation 0.54×10^{-3}/krad, 10^6 daltons, *i.e.*, 72 times lower than for HeLa chromatin (Roti Roti, Mattern, and Cerutti, unpublished results). Factors responsible for these differences in reactivity may include the presence of a large variety of organic scavengers in the nucleoplasm such as non-histone chromosomal proteins (Stein and Thrall, 1973) and differences in the conformation and hydration of the macromolecules between the *in vitro* and *in vivo* situation. In this context it should be kept in mind that the present studies were carried out in low salt. Under these conditions the superstructure of chromatin may differ substantially from that *in situ* in the nucleus (Wagner and Vandegrift, 1972; Wagner and Spelsberg, 1971).

The assay for t′ formation measures a major class of thymine radiation products but not total thymine damage. Since t′ was measured in the trichloroacetic acid precipitable polymeric material, the ring fragments which are spontaneously released from the DNA are not accounted for. An estimate for total thymine ring destruction can be obtained by multiplying the values of t′ by a factor of 8. This factor takes into account the yield of the formation of t′ relative to total thymine destruction and the amount of ring-damaged thymine initially released from the polymer. On this basis a value of 0.3 thymine destroyed per krad in 10^6-dalton DNA is calculated for native HeLa chromatin. This value is higher than that for the formation of single-strand breakage in rat thymus chromatin determined by Ansevin (personal communication) at 0.14 break/krad in 10^6-dalton DNA under comparable conditions. Taking into account that damage is not restricted to thymine but undoubtedly also occurs at the other nucleic acid bases, it follows that base damage is the predominant type of damage induced by γ rays in native chromatin irradiated *in vitro*. While there is little doubt that ring-saturated pyrimidine residues cannot be tolerated in nucleic acids without impairing their biological activity (Rottman and Cerutti, 1966; Remsen and Cerutti, 1972), the relationship between $[^3H]H_2O$ formation from [*Me*-^{3}H]-thymine and the production of biologically important and permanent thymine damage cannot be readily established. Although permanent damage is produced in this reaction, *e.g.*, 5-hydroxymethyl- or hydroperoxymethylthymine (Latarjet *et al.*, 1963), a substantial portion of the 5-methyleneuracil radicals which are the most likely intermediates in this reaction may revert to thymine by chemical repair. Furthermore, 5-hydroxy- or hydroperoxymethyluracil may represent "functionally tolerable damage" (Cerutti, 1974).

Essentially linear dose–response curves were obtained for the formation of $[^3H]H_2O$ and t′ for native chromatin down to doses of 1 krad. If native chromatin does contain, as has been proposed (Clark and Felsenfeld, 1971), substantial portions of naked DNA free of chromosomal proteins much higher initial efficiencies of product formation comparable to that of free DNA would be obtained. This was not observed and our results, therefore, argue against this hypothesis.

Identical dose–response curves were obtained for both reactions of thymine for native and reconstituted chromatin. The two preparations were, therefore, comparable in the structural features which determine the radiation chemical reactivity of the thymine residues. The reactivity of the thymine residues, on the other hand, was considerably lower in nucleoprotein preparations containing only histones or only non-histone chromosomal proteins at ratios comparable to those of HeLa chromatin. This result is particularly striking if it is taken into account that the *total* protein to nucleic acid ratio of these preparations was only about half that of chromatin. It follows, therefore, that the thymine residues in native and fully reconstituted chromatin are more accessible to radical attack than in the partially reconstituted nucleoprotein preparations. It is interesting to refer to some recent models for chromatin structure and function in which it has been speculated that a function of non-histone chromosomal proteins may be to make the DNA available for the binding of RNA polymerase and transcription, perhaps by mediating in a specific manner the association of histones with DNA (Paul, 1972; Stein *et al.*, 1974a,b).

References

Beer, M., Stern, S., Carmalt, D., and Mohlenrich, K. (1966), *Biochemistry 7*, 2283.

Bekhor, I., Kung, G. M., and Bonner, J. (1969), *J. Mol. Biol. 39*, 351.

Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C., Marushige, K., and Tuan, D. (1968), *Science 159*, 47.

Burton, K. (1956), *Biochem. J. 62*, 315.

Burton, K., and Riley, W. T. (1966), *Biochem. J. 98*, 70.

Cerutti, P. (1974), *Naturwissenschaften* (in press).

Clark, R. J., and Felsenfeld, G. (1971), *Nature (London) New Biol. 229*, 101.

Gilmour, R. S., and Paul, J. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9*, 242.

Hariharan, P. V., and Cerutti, P. A. (1971), *Nature (London), New Biol. 229*, 247.

Hariharan, P. V., and Cerutti, P. A. (1972), *J. Mol. Biol. 66*, 65.

Huang, R. C., and Huang, P. C. (1969), *J. Mol. Biol. 39*, 365.

Huberman, J. (1973), *Annu. Rev. Biochem. 42*, 355.

Johansen, I., and Howard-Flanders, P. (1965), *Radiat. Res. 24*, 184.

Kleiman, L., and Huang, R. C. (1972), *J. Mol. Biol. 64*, 1.

Latarjet, R., Ekert, B., and Demersman, P. (1963), *Radiat. Res., Suppl. 3*, 247.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. S. (1951), *J. Biol. Chem. 193*, 265.

Marmur, J. (1963), *Methods Enzymol. 6*, 726.

Okada, S. (1970), Radiation Biochemistry, Vol. I, New York, N. Y., Academic Press.

Paul, J. (1972), *Nature (London) 238*, 444.

Paul, J., and Gilmour, R. S. (1968), *J. Mol. Biol. 34*, 305.

Paul, J., and More, I. R. (1972), *Nature (London), New Biol. 239*, 134.

Pryor, W. A. (1966), Free Radicals, New York, N. Y., Academic Press.

Remsen, J., and Cerutti, P. (1972), *Biochem. Biophys. Res. Commun. 48*, 430.

Roots, R., and Okada, S. (1972), *Int. J. Radiat. Biol. 21*, 329.

Roti Roti, J. L., and Cerutti, P. A. (1974), *Int. J. Radiat. Biol.* (in press).

Rottman, F., and Cerutti, P. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 960.

Stein, G. S., and Borun, T. W. (1972), *J. Cell Biol. 52*, 292.

Stein, G. S., Chaudhuri, S. C., and Baserga, R. (1972), *J. Biol. Chem. 247*, 3918.

Stein, G. S., and Farber, J. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2918.

Stein, G. S., Hunter, G., and Lavie, L. (1974a), *Biochem. J.* (in press).
Stein, G. S., Spelsberg, T. C., and Kleinsmith, L. J. (1974b), *Science 83*, 817.
Stein, G. S., and Thrall, C. L. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 32*, 41.
Swinehart, J. L., Lin, W. S., and Cerutti, P. A. (1974), *Radiat. Res.* (in press).
Van der Schans, G. P., Bleichrodt, J. F., and Blok, J. (1973), *Int. J. Radiat. Biol. 23*, 133.
Wagner, T., and Spelsberg, T. C. (1971), *Biochemistry 10*, 2599.
Wagner, T., and Vandegrift, V. (1972), *Biochemistry 11*, 1431.

Reaction of Cytidine with Ethylating Agents†

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ABSTRACT: The products of the reaction of cytidine and deoxycytidine with stoichiometric amounts of ethyl iodide in anhydrous solution, with or without K_2CO_3, were isolated and characterized. They included 3-ethylcytidine, N^4-ethylcytidine, 3,N^4-diethylcytidine, N^4,N^4-diethylcytidine, and possibly 3,N^4,N^4-triethylcytidine as well as small amounts of unidentified products. The extent of reaction was 25–50%. Data are presented for the ultraviolet absorption spectra and acid dissociation constants of these derivatives. 3-Ethylcytidine, N^4-ethylcytidine, and N^4,N^4-diethylcytidine were obtained after neutral aqueous reaction of cytidine and poly(C) with [^{14}C]-ethyl methanesulfonate and [^{14}C]diethyl sulfate. One of the unidentified derivatives was a major product of the reaction of cytidine with ethyl methanesulfonate and diethyl sulfate. The extent of total ethylation with these reagents did not exceed 0.5%. The reaction of cytidine in poly(dG)·poly(dC) with [^{14}C]ethyl methanesulfonate in neutral aqueous solution was very limited and the products were 3-ethylcytidine and N^4-ethylcytidine in approximately equal amounts. Methylation of cytidine and deoxycytidine with stoichiometric amounts of methyl iodide, in anhydrous solution containing K_2CO_3, led to the almost quantitative formation of 3-methylcytidine only. When the amount of reagent and reaction time were increased, 3,N^4-dimethylcytidine and 3,N^4,N^4-trimethylcytidine were found. Similarly, 3-methylcytidine, 3-ethylcytidine, and N^4-ethylcytidine could be exhaustively methylated or ethylated with the alkyl iodides to form the corresponding derivatives. The direct ethylation of the exocyclic nitrogen of cytidine has not previously been observed in neutral aqueous solution.

Alkylation of cytidine at the 3 position has been shown to be mutagenic in intact TMV (Singer and Fraenkel-Conrat, 1969b) and to cause mispairing when copolymers of cytidine with 3-methylcytidine or 3-ethylcytidine were used as templates by DNA-dependent RNA polymerase (Singer and Fraenkel-Conrat, 1970; Ludlum, 1970; Ludlum and Magee, 1972). The importance of these observations has increased as it has become more apparent that the generally predominant reaction of alkylating agents with the N-7 of guanosine is of little or no importance in mutagenesis or carcinogenesis (Swann and Magee, 1968, 1971; Loveless and Hampton, 1969; Ludlum, 1970; Lijinsky *et al.*, 1972; Goth and Rajewsky, 1972; Kleihues and Magee, 1973; Craddock, 1973).

In the previous paper of this series the reaction of guanosine with several ethylating and methylating agents was examined and it was concluded that the nature of the alkylating group (ethyl *vs.* methyl) played an important role in determining the site and rate of alkylation (Singer, 1972). This approach has been continued in the present study of the alkylation of cytidine in which we present data on the characterization of alkyl derivatives formed after nonaqueous reaction with ethyl iodide and methyl iodide, as well as the detection of products upon alkylation with the less reactive ethyl methanesulfonate ($EtMeSO_3$) and diethyl sulfate (Et_2SO_4) in neutral aqueous solution. To our knowledge, the only previously described derivatives of cytidine found after direct alkylation are 3-methylcytidine (Brookes and Lawley, 1962), 3-benzylcytidine (Brookes *et al.*, 1968), 3-(2-morpholinoethyl)deoxycytidine, and 3,N^4-di(2-morpholinoethyl)deoxycytidine (Price *et al.*, 1968).

The present paper presents data on the isolation and characterization of products of the reaction of cytidine and ethyl iodide in nonaqueous media. These were 3-ethylcytidine, N^4-ethylcytidine, 3,N^4-diethylcytidine, N^4,N^4-diethylcytidine and possibly 3,N^4,N^4-triethylcytidine. The products of the reaction of cytidine, poly(C), and poly(dG)·poly(dC) in aqueous solution at neutrality with ^{14}C-labeled diethyl sulfate and ^{14}C-labeled ethyl methanesulfonate were found to include 3-ethylcytidine, N^4-ethylcytidine, and N^4,N^4-diethylcytidine. The relationship of the new finding, that the amino group of cytidine is alkylated at neutrality, to possible biological effects is discussed.

Experimental Section

Reaction of Cytidine with Alkyl Iodides. (a) One-hundred milligrams of cytidine or deoxycytidine was dissolved in 1 ml of dimethyl sulfoxide containing 60 mg of anhydrous K_2CO_3. Alkyl iodide (25 μl) was added and the mixture was stirred at room temperature. After 2 hr, 20 mg of K_2CO_3 and 10 μl of alkyl iodide were added. Stirring was continued for an additional 2 hr. The reaction mixtures were filtered through a 2-cm Celite column in a Pasteur pipet. The filtrate was then subjected to electrophoresis and chromatography to separate the reaction products. (b) The reaction conditions

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