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# EFFECT OF THE ALKYLATING AGENT TRISETHYLENEIMINOBENZOQUINONE (TRENIMON) ON THE TEMPLATE ACTIVITY OF CHROMATIN AND DNA IN RNA AND DNA POLYMERASE SYSTEMS

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**Abstract**—Treatment of chromatin from Ehrlich ascites tumor cells with the alkylating antitumor agent 2,3,5-trisethyleneiminobenzoquinone-1,4 (Trenimon) decreases its template activity in a RNA polymerase system. The inhibition in the template activity of the chromatin is greater after treatment *in vivo* than after treatment of isolated chromatin with an equivalent concentration *in vitro*. Deproteinized DNA isolated after *in vivo* treatment with the alkylating agent shows the same inhibition in template activity as DNA treated *in vitro* with a corresponding dose. Isolated chromatin treated *in vitro* exhibits the same sensitivity to the alkylating agent as deproteinized DNA. The difference between the *in vivo* and the *in vitro* treated chromatin preparations is not due to an increased ribonuclease activity of the chromatin treated *in vivo*. Treatment of tumor cells with the alkylating agent at concentrations which inhibit the template activity of the chromatin decreases the capacity of the cells for the puromycin insensitive uptake of acetate into acid soluble nuclear protein. The observed inhibition in the template activity of chromatin in the RNA polymerase system after *in vivo* treatment with the alkylating agent is interpreted as a modification of the nucleoprotein structure which is not explained by an alkylation of DNA alone.

Under the conditions used in our experiments the *in vivo* as well as the *in vitro* treatment of the chromatin with the alkylating trisethyleneimino compound Trenimon does not lead to an inhibition of its template activity in a DNA polymerase system.

NUMEROUS investigators have demonstrated that alkylating antitumor agents inhibit the incorporation of radioactive substrates into DNA and RNA of tumor cells, these studies have been reviewed by Wheeler.[1,2] However, the detailed mechanism leading to this inhibition is still obscure.

It is well established that alkylating agents interact with DNA under *in vivo* as well as under *in vitro* conditions (review by Wheeler[2]). Based on these studies it has been postulated that the inhibitory effect of the alkylating agents is caused by an alkylation of DNA.[3] However, this conclusion has been questioned by many authors.[4–9]

Within the tumor cell DNA is complexed with nuclear proteins. Evidence is accumulating that the predominant regulation of RNA and DNA synthesis occurs by mechanisms which affect the deoxyribonucleoprotein complex.[10,11] It is possible, therefore, that the impairment of nucleic acid synthesis is not simply due to an alkylation of DNA but caused by a more complicated modification of the nucleoprotein structure.

Methods are available now for the preparation of chromatin with preservation of its biological activity.[12,13] It seemed interesting, therefore, to investigate the effect of an alkylating agent on the template activity of the biological deoxyribonucleoprotein complex in RNA and DNA polymerase systems.

Previous studies by Ruddon and Johnson[14] on the effects of *N*-mustard on the template activity of DNA and a deoxyribonucleoprotein prepared from *E.coli* in a RNA polymerase system had indicated that the nucleoprotein complex is several orders of magnitude less sensitive to the alkylating agent than deproteinized DNA. However, with regard to the profound differences between bacterial and mammalian chromatin it seemed necessary to study the sensitivity of the template activity of chromatin from tumor cells to an alkylating antitumor agent.

In this paper it is shown that isolated chromatin of Ehrlich ascites tumor cells is as sensitive to an *in vitro* treatment with the alkylating ethyleneimino compound Trenimon as deproteinized DNA. Chromatin from ascites cells treated *in vivo* leads to a markedly stronger inhibition in the template activity of the chromatin in an RNA polymerase system than treatment of isolated chromatin *in vitro*.

Evidence is presented that the inhibition of the template activity of the chromatin observed after *in vivo* treatment cannot be sufficiently explained by an alkylation of DNA. The inhibition in the template activity observed after *in vivo* treatment is discussed in terms of a modification of the nucleoprotein structure produced by an interference of the alkylating agent with metabolic reactions engaged in chromatin metabolism. It is demonstrated that concentrations of the alkylating agent which inhibit the template activity of the chromatin decrease the acetylation of the acid soluble nuclear proteins.

The template activity of the chromatin in a DNA polymerase system proved to be resistant to extremely high concentrations of the alkylating agent.

## MATERIALS AND METHODS

Ehrlich ascites tumor cells were grown and harvested as described previously.[15] 2,3,5-trisethyleneiminobenzoquinone-1,4 (Trenimon) was donated by Farbenfabriken Bayer AG, Germany. (Methyl-$^3$H)TTP (10 c/m-mole) was obtained from Schwarz Bioresearch, Inc., Orangeburg, N.Y., U.S.A.; adenosine-8-$^{14}$C-triphosphate (40 mc/m-mole), $^3$H-sodium acetate (500 mc/m-mole) and $^{14}$C-lysine (140 mc/m-mole) were purchased from Amersham, Buckinghamshire, England. All nonlabeled ribo- and deoxyribonucleoside triphosphates were obtained from Sigma Chemical Corp., St. Louis, Mo., U.S.A.

DNA was extracted from Ehrlich ascites tumor cells according to the procedure of Thomas *et al.*[16] Chromatin was prepared from ascites cells following the method described by Marushige and Bonner.[17]

RNA polymerase (fraction IV) was prepared from *E. coli* cells according to the procedure described by Chamberlin and Berg.[18]

The assay contained in a total volume of 0·25 ml in $\mu$moles: Tris/HCl (pH 7·9) 10·0; NaCl 40·0; mercaptoethanol 3·0; $MnCl_2$ 0·25; $MgCl_2$ 1·0; CTP, UTP, GTP, ATP 0·004 each; 0·5 $\mu$c ATP-8-$^{14}$C; 12 $\mu$g of enzyme protein and native DNA or chromatin as indicated. Samples were incubated for 30 min at 37°. The reaction was stopped by the addition of 5 ml 5% trichloroacetic acid and the resulting precipitate collected on Whatman GF/C filter discs. The discs were washed three times

with 5% trichloroacetic acid, air dried, suspended in 10 ml scintillation fluid (naphthalene 738 g; 2,5-diphenyloxazol 46·0 g; 2-(1-naphthyl)-5-phenyloxazol 0·46 g; xylene 3500 ml; dioxane 3500 ml; ethanol 2100 ml) and counted in a scintillation spectrophotometer.

DNA polymerase was prepared as described previously.[19] For the assay a modification of the system described by Smellie *et al.*[20] was used. The system contained in a total volume of 1·0 ml in μmoles: Tris/HCl (pH 7·9) 40·0; $MgCl_2$ 5·0; mercaptoethanol 1·0; dATP, dGTP, dCTP, dTTP 0·005 each; 0·5 μc (methyl-$^3$H)dTTP; ATP 5·0; various concentrations of DNA or chromatin as indicated and about 20 mg of enzyme protein. The reaction mixture was incubated for 30 min at 37°. Reaction was stopped by the addition of 4·0 ml of 0·7 N $HClO_4$. The precipitate was washed three times with 5 ml 0·2 N $HClO_4$, dissolved in 0·5 ml of "Nuclear Chicago Solubilizer" (NCS). Ten ml of the scintillation fluid described above were added and the solution counted in a scintillation spectrophotometer.

Preparation of acid soluble nuclear proteins: ascites cells were washed two times in 10 vol. 0·25 M sucrose at 4° by suspension and subsequent centrifugation at 750 *g*. The pellet after the final washing was resuspended in 12 vol. of 0·01 M Tris (pH 7·5). After 30 min the cells were homogenized in a Dounce type homogenizer with 10 strokes of the tightly fitting pestle. The homogenate was centrifuged for 5 min at 750 *g*. The pellet was resuspended in 0·25 M sucrose by repeated vigorous expulsion from a 10 ml Mohr pipette until a homogeneous suspension was achieved. The suspension was centrifuged for 5 min at 750 *g*, the pellet resuspended in 0·25 M sucrose as described above and again centrifuged for 5 min at 750 *g*. The sediment was suspended in 10 vol. of 0·14 M NaCl–0·01 M sodium citrate and again centrifuged as above. The pellet was then homogenized in 10 vol. of the NaCl–sodium citrate solution with the aid of a teflon pestle fitting into the centrifuge tube. The suspension was stirred for 10 min in an ice bath and centrifuged for 10 min at 12,000 *g*. The extraction with NaCl–sodium citrate solution was repeated two more times. The final pellet was homogenized in 2 M NaCl with the aid of a teflon pestle. The resulting viscous solution was stirred in an ice bath for 45 min and centrifuged at 22,000 rev/min in a rotor No. 40 of a Beckman ultracentrifuge. The supernatant was decanted and acidified with $H_2SO_4$ to a final concentration of 0·25 N.

The suspension was stirred for 20 min and the precipitate collected by centrifugation (10 min at 12,000 *g*). The precipitate was reextracted with 0·25 N $H_2SO_4$ as described above. The combined supernatants contained the acid soluble nuclear proteins operationally called "histones". The histones were precipitated by the addition of trichloroacetic acid to a final concentration of 20% and collected by centrifugation. The pellet was washed with acid acetone (0·1 ml conc. HCl per 200 ml acetone) and finally dried in desiccator. For the determination of the protein content and the radioactivity the material was solubilized in a small volume of 0·25 N $H_2SO_4$. The residue remaining after extraction of the acid soluble nuclear proteins was solubilized in 10 vol. of 0·1 N NaOH. This solution contained the so called nonhistone chromosomal proteins.

Protein determinations were performed according to Lowry *et al.*[21] with bovine serum albumin as standard.

DNA was determined by using the modification of the Ceriotti procedure described by Keck[22] with calf thymus DNA as standard.

## RESULTS

Table 1 shows the properties of the RNA polymerase system in the presence of chromatin from Ehrlich ascites tumor cells. As can be seen, the presence of all four ribonucleoside triphosphates is required for full activity. Chromatin of ascites cells shows about 30 per cent of the template activity of native deproteinized DNA.

TABLE 1. REQUIREMENTS FOR AMP INCORPORATION INTO RNA DIRECTED BY CHROMATIN FROM EHRLICH ASCITES TUMOR CELLS

| | $\mu\mu$moles AMP incorporated | Per cent |
|---|---|---|
| Complete system | 61·2 | 100 |
| Minus enzyme | 6·1 | 10 |
| Minus chromatin | 1·8 | 3 |
| Native DNA (2·5 $\mu$g) instead of chromatin | 192·1 | 314 |
| Minus GTP | 17·3 | 28 |
| Minus UTP | 11·0 | 18 |
| Minus CTP | 11·7 | 19 |
| Minus GTP, UTP, CTP | 14·4 | 23 |

The complete system is described under methods. The chromatin containing samples received 10·0 $\mu$g of chromatin = 2·5 $\mu$g DNA.

Figure 1a shows the effect of trisethyleneiminobenzoquinone (Trenimon) on the template activity of chromatin from Ehrlich ascites tumor cells in the RNA polymerase system. As can be seen, treatment of chromatin *in vivo* leads to a markedly stronger inhibition of the template activity than a treatment of isolated chromatin *in vitro*. The *in vitro* system contained the same amount of alkylating agent per liter of the assay medium as was applied per kilogram tumor bearing animal under *in vivo* conditions.

To determine if the effective concentration of the alkylating agent *in vivo* is comparable to the concentration achieved in the *in vitro* system, the template activity of DNA isolated after *in vivo* treatment of ascites cells was compared with the template activity of DNA treated *in vitro*. In accordance with previous studies from this laboratory[2,3] relatively high concentrations of the alkylating agent are required before a clearcut effect can be observed. Figure 1b demonstrates, however, that the inhibition of the template activity of DNA treated *in vivo* with a given dose per kg is within the same range as the inhibition observed after treatment of isolated DNA *in vitro* containing the same dose per liter of the incubation medium. Thus, the difference observed between *in vivo* and *in vitro* treated chromatin preparations cannot be explained by differences in the effective concentration of the alkylating agent to which the chromatin had been exposed.

A comparison of Fig. 1a with Fig. 1b demonstrates that the extent of the inhibition of the template activity of *in vitro* treated chromatin is close to the inhibition produced by a treatment of native deproteinized DNA from ascites cells. Thus, the

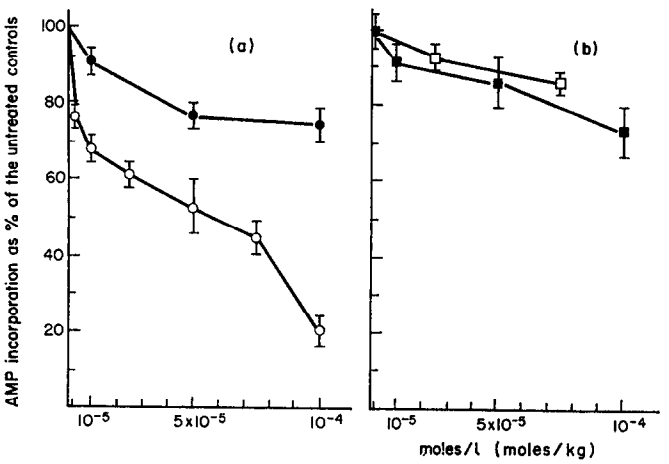


FIG. 1. Effect of trisethyleneiminobenzoquinone (Trenimon) on the template activity of chromatin and DNA in a RNA polymerase system.

(a) Chromatin treated *in vitro* ●—●—● (1 μg DNA per assay)
Chromatin treated *in vivo* ○—○—○ (1 μg DNA per assay)

(b) DNA treated *in vitro* ■—■—■ (1 μg DNA per assay)
DNA treated *in vivo* □—□—□ (1 μg DNA per assay)

For *in vitro* treatments 6 ml samples of the chromatin solution in 0·01 M Tris/HCl (pH 7·4) containing 1 mg chromatin per ml were incubated at 37° in the presence of various concentrations of the alkylating agent as indicated. After 1 hr the solution was dialyzed against 10 l. of 0·01 M Tris/HCl (pH 8·0). *In vitro* treatment of DNA was performed as described previously.[23] *In vivo* treatments were performed by injecting an aqueous solution of the alkylating agent i.p. into tumor bearing mice 1 hr before harvesting the cells. Template activity was measured by the incorporation of $^{14}$C-AMP into RNA using the RNA polymerase system described under methods. Each point represents the average of six determinations. The values are expressed as the mean per cent change from control ± S.E.M. Except for the treatment with the alkylating agent, the controls were handled exactly like the corresponding assays exposed to the drug.

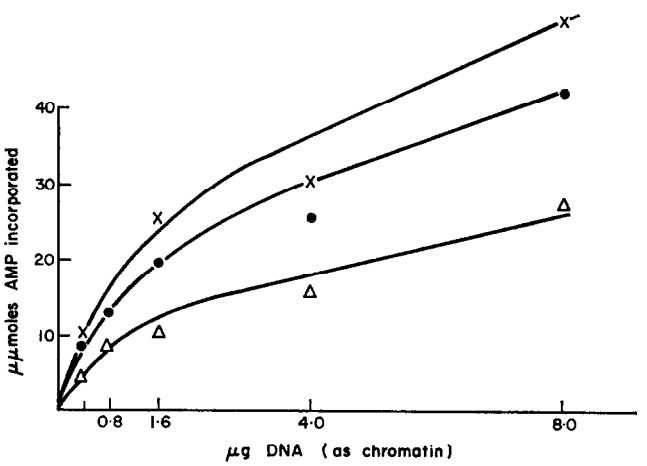


FIG. 2. Effect of trisethyleneiminobenzoquinone on the template activity of DNA in a RNA polymerase system as a function of template concentration. ×—×—× control, ●—●—● + 1·2 × $10^{-6}$moles/kg Trenimon, △—△—△ + 2·4 × $10^{-5}$moles/kg Trenimon. Tumor bearing mice were injected i.p. with the alkylating agent 1 hr before harvesting the cells. Template activity was determined in the RNA polymerase assay described under methods.

proteins of mammalian chromosomes do not seem to have a protective effect against the alkylating agent as has been suggested by Ruddon and Johnson[14] with regard to their studies using a bacterial nucleoprotein complex.

Figure 2 shows the incorporation of AMP into RNA in the presence of increasing amounts of *in vivo* treated and untreated chromatin. As can be seen, the percent inhibition observed after treatment with the alkylating agent remains relatively constant and is independent of the amount of template present in the assay.

Figure 1 demonstrates that in the presence of chromatin treated *in vivo* less RNA is synthesized than in the presence of chromatin treated with a corresponding dose *in vitro*. In order to investigate if this difference is due to a higher ribonuclease activity of the chromatin prepared after *in vivo* treatment, the ribonuclease activity of this preparation was compared to the activity of an untreated control. The ribonuclease activity was measured by following the loss of acid soluble radioactivity from $^{14}C$ labeled RNA. $^{14}C$-RNA was synthesized in the *in vitro* RNA polymerase system using calf thymus DNA as template. Table 2 shows that the total radioactivity recovered in

TABLE 2. STABILITY OF RNA IN PRESENCE OF CHROMATIN FROM CELLS TREATED WITH TRISETHYLENEIMINOBENZOQUINONE AND CHROMATIN FROM UNTREATED CELLS

| Chromatin source | $^{14}C$-RNA recovered in the acid insoluble fraction (dis./min/assay) |
|---|---|
| Untreated cells | 22,075 |
| | 21,168 |
| | 27,644 |
| Treated cells | 29,272 |
| | 21,918 |
| | 30,975 |

Radioactive RNA was synthesized in the RNA polymerase system described under methods in presence of 10 $\mu$g native calf thymus DNA, with $^{14}C$-ATP as the labeled substrate. After 45 min at 37° the incubation was stopped by heating in a boiling water bath for 2 min. After 10 min cooling in an ice bath the samples received 100 $\mu$g of either treated or untreated chromatin and the assays were incubated at 37° for another 45-min period. Incubation was stopped by the addition of 5 ml of icecold 5% trichloroacetic acid. The precipitate was collected on Whatman GF/C filter discs and washed three times on the filter with 10 ml of 5% trichloroacetic acid. The filters were air dried and the radioactivity determined in the liquid scintillation system described for the RNA polymerase assay. Each determination was performed in triplicate. Tumor cells were treated by injecting 5 $\mu$moles/kg trisethyleneiminobenzoquinone i.p. into tumor bearing mice 1 hr before harvesting the cells.

the acid insoluble fraction is the same if the $^{14}C$ labeled RNA is incubated with the chromatin treated *in vivo* or in presence of untreated chromatin. The RNA production in the assay containing the chromatin treated *in vitro* with the equivalent concentration of the alkylating agent, is close to the control value. The smaller amount of RNA formed in the presence of chromatin from Trenimon treated cells—compared to the amount of RNA synthesized in the presence of chromatin treated *in vitro* with the alkylating agent—is therefore not due to an increased degradation of RNA.

As shown in Table 3 there is also no change in the gross protein composition of the chromatin after treatment of the tumor cells with the alkylating agent, except for a slight increase in the amount of the acid soluble protein (operationally called histones) at high concentrations of the alkylating agent.

TABLE 3. EFFECT OF TRISETHYLENEIMONOBENZOQUINONE ON THE PROTEIN COMPOSITION OF CHROMATIN FROM EHRLICH ASCITES TUMOR CELLS

| Dose (moles/kg) | Histone (mg/mg DNA) | Nonhistone protein (mg/mg DNA) | Nonhistone/histone |
|---|---|---|---|
| — | 2·1 | 1·1 | 0·52 |
| $1·5 \times 10^{-6}$ | 2·1 | 1·1 | 0·52 |
| $3·0 \times 10^{-6}$ | 2·0 | 1·1 | 0·55 |
| $7·5 \times 10^{-6}$ | 2·0 | 1·0 | 0·50 |
| $1·5 \times 10^{-5}$ | 2·0 | 1·0 | 0·50 |
| $3·0 \times 10^{-5}$ | 2·4 | 1·0 | 0·42 |
| $6·0 \times 10^{-5}$ | 2·6 | 1·2 | 0·46 |

Tumor bearing mice were injected i.p. 1 hr before harvesting the ascites cells. Each determination was performed on the pooled tumor cells of 3 mice. Extraction of nuclear proteins was performed as described under methods.

There is evidence for a positive correlation between histone acetylation and RNA synthesis.[24] In search of an explanation for the stronger inhibition of the template activity by the *in vivo* treatment compared to the *in vitro* treatment of the chromatin, the effect of the alkylating agent on the acetylation of the histones was studied. As a measure of histone acetylation we followed the uptake of radioactivity from $^{3}H$-acetate into acid soluble nuclear proteins. In contrast to the behavior of $^{14}C$-lysine the incorporation of labeled acetate into the acid soluble nuclear protein is insensitive to puromycin (Table 4). This is in accordance with the findings of other authors[25,26] and indicates a modification of the protein at the polymer level. Using the assay described in the legend to Table 4 incorporation of radioactivity from $^{3}H$-acetate into acid soluble nuclear proteins proceeds linear with time for more than 60 min. Figure 3 demonstrates the effect of an *in vivo* treatment with the alkylating agent on the uptake of radioactivity from $^{3}H$-acetate into the acid soluble nuclear proteins. As can be seen, a treatment with $10^{-6}$moles/kg trisethyleneiminobenzoquinone which just begins to show an inhibition of the template activity of the chromatin in the RNA polymerase system also causes a decrease in the uptake of $^{3}H$-acetate into the acid soluble nuclear proteins.

TABLE 4. EFFECT OF PUROMYCIN ON THE INCORPORATION OF $^{3}$H-ACETATE AND $^{14}$C-LYSINE INTO ACID SOLUBLE NUCLEAR PROTEIN OF EHRLICH ASCITES TUMOR CELLS

| Experiment No. | Addition | Labeled precursor | Dis./min/mg protein/hr | Per cent |
|---|---|---|---|---|
| | — | $^{3}$H-acetate | 5724 | 100 |
| 1 | Puromycin | $^{3}$H-acetate | 5097 | 89 |
| | — | $^{14}$C-lysine | 3557 | 100 |
| | Puromycin | $^{14}$C-lysine | 159 | 4 |
| | — | $^{3}$H-acetate | 6135 | 100 |
| 2 | Puromycin | $^{3}$H-acetate | 6810 | 111 |
| | — | $^{14}$C-lysine | 2749 | 100 |
| | Puromycin | $^{14}$C-lysine | 222 | 8 |

1 ml of ascites cells were incubated in 10 ml of a medium consisting of 50% Krebs-Ringer solution containing 0·1 M Tris/HCl (pH 7·4) and 50% ascites serum. Glucose was added to a final concentration of 10 mg/ml. $^{3}$H-acetate was added at 25 $\mu$c/ml and $^{14}$C-lysine at 0·2 $\mu$c/ml where indicated. The samples were incubated for 1 hr at 37°. For the measurement of the $^{3}$H-acetate incorporation the assays were stopped by the addition of unlabeled Na-acetate to a final concentration of 0·01 M, centrifuged and the sediment washed 2 times with cold 0·25 M sucrose. The samples containing $^{14}$C-lysine were stopped by centrifugation and resuspension of the cells in cold 0·25 M sucrose and 2 subsequent washings in the same medium. Puromycin concentration was 100 $\mu$g/ml.

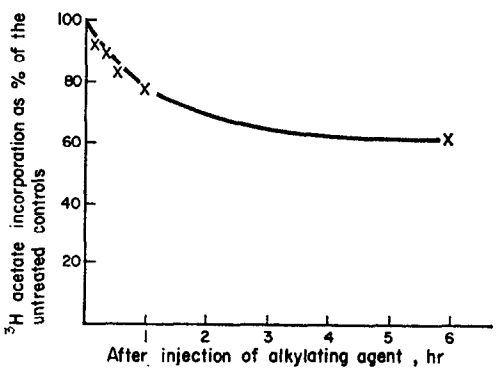


FIG. 3. Effect of trisethyleneiminobenzoquinone on the uptake of $^{3}$H-acetate into acid soluble nuclear protein of Ehrlich ascites tumor cells. Tumor bearing mice were injected i.p. with $10^{-6}$moles/kg of the alkylating agent before harvesting the cells. $^{3}$H-acetate incorporation was measured as described in the legend to Table 4.

The effects of trisethyleneiminobenzoquinone on the template activity of chromatin and DNA in a DNA polymerase system are pictured in Figs. 4a and b. The properties of the system are shown in Table 5. DNA synthesis was followed by the uptake of radioactivity from (methyl-$^{3}$H)dTTP into an acid insoluble, DNase sensitive product. As can be seen, the incorporation requires the presence of either native or heat denatured DNA. All four deoxyribonucleoside triphosphates are required for full activity. With chromatin as template, the activity of the system is very low but still dependent on the presence of all four deoxyribonucleoside triphosphates

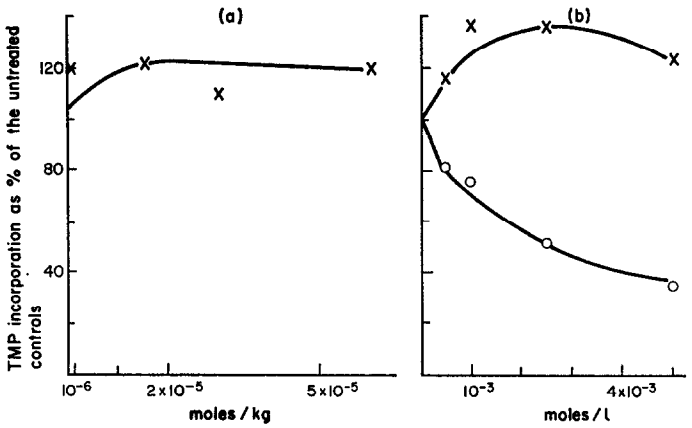


FIG. 4. Effect of trisethyleneiminobenzoquinone (Trenimon) on the template activity of chromatin and DNA in a DNA polymerase system.

(a) Chromatin treated *in vivo*; tumor bearing mice were injected i.p. with various doses of the alkylating agent as indicated 1 hr before harvesting the cells.

(b) Chromatin treated *in vitro* ×—×—×, heat denatured DNA treated *in vitro* ○—○—○. *In vitro* treatments were performed as described in the legend to Fig. 1. Template activity was measured by the uptake of $^3$H-TMP into DNA using the DNA polymerase assay described under methods. The DNA content in the chromatin containing assays were 85 μg. The DNA containing samples received 40 μg heat denatured DNA per assay. Each point represents the average of 3 determinations.

TABLE 5. REQUIREMENT FOR TMP INCORPORATION INTO DNA DIRECTED BY DNA OR CHROMATIN FROM EHRLICH ASCITES TUMOR CELLS

| | μμmoles TMP incorporated | Per cent |
|---|---|---|
| Complete system<br>Template: den. DNA (100 μg) | 206·1 | 100 |
| Complete system<br>Template: native DNA (100 μg) | 264·3 | 130 |
| Minus dATP (template: den. DNA) | 58·5 | 28 |
| Minus dCTP (template: den. DNA) | 103·8 | 50 |
| Minus dGTP (template: den. DNA) | 82·8 | 40 |
| Minus dATP, dGTP, dCTP<br>(template: den. DNA) | 28·8 | 14 |
| Plus DNase | <0·1 | <0·05 |
| Complete system<br>Template: chromatin (40 μg DNA) | 3·87 ± 0·34* | 100 |
| Minus dATP (template: chromatin) | 2·68 ± 0·29* | 70 |
| Minus dGTP (template: chromatin) | 1·92 ± 0·56* | 41 |
| Minus dCTP (template: chromatin) | 1·57 ± 0·47* | 49 |
| Minus dATP, dCTP, dGTP<br>(template: chromatin) | 0·017 ± 0·03 | 4 |

The complete system is described under methods. The assay plus DNase contained 0·8 mg deoxyribonuclease of bovine pancreas.

* Mean of six determinations ± S.D.

for full activity. Figure 4 demonstrates that *in vivo* as well as *in vitro* treatment of chromatin with the alkylating agent does not lead to an inhibition in the chromatin directed incorporation of dTMP into DNA. With very high concentrations of the alkylating agent—which can be only applied *in vitro*—there is even an increase in the template activity of chromatin. The template activity of deproteinized DNA is impaired by trisethyleneiminobenzoquinone. However, a significant effect can only be observed after treatment with doses which are three to four orders of magnitude greater than those causing an inhibition of the uptake of thymidine into DNA of intact cells.[27]

## DISCUSSION

The results presented here demonstrate that in contrast to the behavior of deoxyribonucleoprotein from *E.coli* the template activity of chromatin from Ehrlich ascites cells in a RNA polymerase system is as sensitive to alkylation as the template function of deproteinized DNA.

It is shown furthermore, that treatment of chromatin from Ehrlich ascites cells with the alkylating agent *in vivo* leads to a stronger inhibition of the template activity in the RNA polymerase system than treatment of isolated chromatin *in vitro*. No significant difference between *in vivo* and *in vitro* treatment was observed when the effect of the alkylating agent on the template activity of deproteinized DNA was studied. It must be concluded, therefore, that the inhibition in the template activity of the chromatin observed after treatment with the alkylating agent *in vivo* is not simply due to an alkylation of DNA.

It is demonstrated that the differences observed cannot be explained by an increased breakdown of RNA in presence of the *in vivo* treated chromatin. One has to conclude, therefore, that treatment of Ehrlich ascites cells with the alkylating agent results in another as yet unknown modification of the nucleoprotein complex. As this modification is observed only under *in vivo* conditions, it is probably caused by an interference with enzymatic reactions involved in nucleoprotein metabolism.

The mechanism of this modification is still unclear. No change in the histone or nonhistone protein content of the chromatin could be observed after treatment of the tumor cells with concentrations of the alkylating agent which cause an inhibition in the template activity. Evidence has been presented for a correlation between histone acetylation and RNA synthesis.[24] With regard to these studies it is tempting to correlate the observed decrease in the uptake of $^{3}$H-acetate into basic nuclear protein with the inhibition of the template activity in the RNA polymerase system. However, more studies especially on the quantitative relationship between histone acetylation and template activity of chromatin are required before a definite evaluation of the observed effects can be made.

It has been demonstrated that treatment of tumor cells with alkylating agents results in an increased binding of protein to DNA which cannot be removed by standard phenol extraction procedures.[28–31] Preliminary experiments indicate that this effect is not the result of DNA–protein crosslinks caused by the bi- or trifunctional alkylating agents, as assumed previously,[29,30] but due to effects on nucleoprotein metabolism. Further experiments will show if there is any correlation between this binding of protein to DNA and the observed effects on the template activity of the chromatin in the RNA polymerase system.

Numerous authors have demonstrated that the uptake of labeled substrates by intact cells into DNA is inhibited by treatment with alkylating agents to a greater extent than the incorporation of radioactive precursors into RNA.[32–35] Evidence has been presented that these effects are not sufficiently explained by an inhibition in the polymerizing enzymes or the formation of the nucleoside triphosphates.[14,35]

In contrast to these studies in the *in vitro* system used here, the template activity of the chromatin in the RNA polymerase assay proved to be more sensitive to the alkylating agent than the template activity for DNA synthesis. However, the present results are in agreement with reports of other authors who investigated the effects of alkylating agents on the template activities of deproteinized DNA in RNA and DNA polymerase systems.[14,36] Because of the greater sensitivity of the RNA polymerase system it has been hypothesized that the impairment of DNA synthesis is the result of a preceding inhibition of a messenger RNA required for the replication of DNA.[14] It should be mentioned, however, that the biological function of the DNA polymerases used in all these studies including our own is uncertain. It is well possible that all the DNA polymerases used so far are repair enzymes with primer and template requirements which differ from those of the replicating enzymes.

Under the conditions reported here, about $10^{-6}$moles/kg of the trisethyleneiminobenzoquinone are required to produce a measurable decrease in the template activity in the RNA polymerase system. This dose is approximately two times higher than that which is necessary to inhibit the multiplication of the ascites cell line used in our studies by a single injection of the alkylating agent. However, the measurement of the overall template activity is probably not a very sensitive indicator for the functional integrity of the template. The studies presented here point to an interference of the alkylating agent with metabolic reactions which in turn cause a modification of the biological activity of the nucleoprotein complex. It is conceivable that these modifications of the nucleoprotein complex result not only in a decrease in the amount of RNA synthesized but also in qualitative changes in the RNA formed in the presence of the modified template. It seems probable that this latter effect occurs at lower concentrations of the alkylating agent and may be of greater biological importance than the decrease in overall RNA synthesis. Experiments have been initiated to characterize the RNA synthesized in presence of the *in vivo* treated chromatin in comparison to the RNA formed in the presence of an untreated template.

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