Reaction of Dihydropyrrolizines with Deoxyribonucleic Acids *in vitro*

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1. Thermal denaturation profiles of *Escherichia coli* DNA pretreated with monocrotaline pyrrole *in vitro* showed no difference from control DNA samples during heating. A substantial increase in the degree of renaturation during cooling of the pretreated DNA samples was observed. The degree of renaturation was dependent on the concentration of pyrrole used. 2. When rat liver DNA was pretreated with monocrotaline pyrrole there was a greater degree of renaturation after heat treatment than was found with *E. coli* DNA. 3. Equilibrium-density-gradient centrifugation in alkaline caesium chloride showed that DNA pretreated with monocrotaline pyrrole and heat denatured, renatured to a greater extent on quenching in ice than did untreated control DNA. The degree of renaturation was similar whether the initial treatment of the DNA with pyrrole was for 1 or for 15 min. The reaction also appeared to be independent of pH between 5.5 and 9.0. 4. Retrorsine pyrrole was as effective as monocrotaline pyrrole in cross-linking the DNA, but monocrotaline pyrrole exposed to water for 5 min before the addition of the DNA was ineffective. Pretreatment of *E. coli* DNA with synthetic bis-hydroxymethylpyrrole esters also caused renaturation after heat treatment. Monoesters were ineffective. 5. After treatment of rats with retrorsine, no cross-linking of the liver DNA could be demonstrated.

In addition to acute hepatotoxic effects, the administration of some pyrrolizidine alkaloids, such as heliotrine, to rats results in long-term changes in the liver characterized by the appearance of large bizarre cells (Schoental & Magee, 1959; Jago, 1970). Monocrotaline can also cause cytological changes in the lungs and kidneys of rats (Hayashi & Lalich, 1967). It has been suggested (Mattocks, 1968; Culvenor *et al.*, 1969) that cytological changes may be due to dihydropyrrolizine (pyrrolic) metabolites of the alkaloids produced in the liver.

The long-term inhibition of liver cell mitosis led Black & Jago (1970) to investigate the interaction of the pyrrolic dialcohol, dehydroheliotridine (compound III) with DNA *in vitro*. They showed that an interaction occurred at room temperature under mildly acidic conditions or at 100°C at neutral pH values.

Cyclic dihydropyrrolizine diesters such as monocrotaline pyrrole or retrorsine pyrrole (compounds I and II) are considerably more reactive to neutrophiles than the dihydropyrrolizine alcohol (compound III) used by Black & Jago (1970). The diesters prepared chemically and injected intravenously into rats produce liver or lung lesions similar to those described for the parent alkaloids (Butler *et al.*, 1970).

In the present paper we describe some of the actions of monocrotaline pyrrole and retrorsine pyr-

role on DNA *in vitro* at room temperature and at neutral pH values.

Materials and Methods

Materials

Chemicals. Chemicals (AnalaR grade) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. unless otherwise stated. Caesium chloride (optical grade, Mann Research Laboratories, New York, N.Y., U.S.A.) was used without further purification.

DNA. *Escherichia coli* and *Micrococcus lysodeikticus* DNA were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and *Clostridium perfringens* DNA from the Worthington Biochemical Corp., N.J., U.S.A. Rat liver nuclei were prepared from normal or treated rats by the method of Widnell & Tata (1964). The DNA was extracted from the nuclear preparation by the method of Mattoccia & Cummings (1971). After the final dialysis, the DNA was dried at 20°C *in vacuo*. Stock solutions of DNA (1 mg/ml) were prepared in 0.014M-NaCl-0.0015M-trisodium citrate buffer, pH 7.0. Solutions of DNA were dialysed for 18 h against this medium before use. All dialysis tubing was boiled in 10mM-EDTA, adjusted to pH 7.0 with

0.01M-NaOH, before use and then thoroughly washed in distilled deionized water.

Dihydropyrrolizine derivatives. Monocrotaline and retrorsine pyrroles were prepared from the parent alkaloids by the method of Mattocks (1969) and then were dissolved in freshly redistilled 1,2-dimethoxyethane. *N*-methyl-2-hydroxymethylpyrrole was prepared from *N*-methylpyrrole-2-aldehyde (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) by the method of Ryskiewicz & Silverstein (1954). It was converted into the *N*-ethyl carbamate ester by the action of ethyl isocyanate (Koch-Light Laboratories Ltd.) under reflux. The carbamate, recrystallized from light petroleum (b.p. 60–80°C), had m.p. 46°C (corr.). Solutions of these were prepared in 1,2-dimethoxyethane. This solvent was chosen, as the pyrrolic derivatives readily dissolved in it without any chemical interaction and such solutions were miscible with water.

Interaction of pyrrolic derivatives with DNA

To 200 µg of DNA in 0.5 ml of buffer, usually 0.04M-tris adjusted to pH 7.4 with 1M-HCl at 20°C, was added 0.31–1.55 µmol of pyrrole solution. The maximum volume of pyrrole solution added was 50 µl. The contents of the tube were mixed and after a fixed time-interval, normally 5 min, 1.5 ml of ethanol at 0°C were added. Precipitated DNA was collected on a glass rod and immediately redissolved in 0.014M-NaCl–0.0015M-trisodium citrate buffer, pH 7.0. The solution was dialysed for 18 h at 4°C against this buffer. For controls, 1,2-dimethoxyethane was added in equivalent volumes to solutions of DNA that were subsequently treated in a similar manner. After dialysis, E_{260} of the non-diffusible material was determined.

Denaturation of DNA

By heat. The dialysed DNA solutions (10–20 µg/ml) obtained as described above were heated at 100°C for 5 min and were then quenched in ice for 10 min.

By alkali. *M. lysodeikticus* DNA, to be used as a density marker for analytical ultracentrifuge studies, was prepared by adjusting a stock solution (100 µg/ml in 0.014M-NaCl–0.0015M-trisodium citrate buffer, pH 7.0) to pH 12.0 with 0.01M-NaOH. This was left for 2 min at 20°C and then 10 µl was transferred directly to the buffered caesium chloride sample to be centrifuged.

Equilibrium-density-gradient centrifugation

To 0.9 ml of caesium chloride (relative density 1.80) in 0.04M-Na₂CO₃–NaHCO₃ buffer, pH 10.8, was added 0.5–1 µg of the marker, either *Cl. perfringens* or alkali-denatured *M. lysodeikticus* DNA, and 1–3 µg of DNA sample. If necessary the relative density of

the mixture was adjusted to 1.71 with water or solid caesium chloride. Centrifugation was for 20 h at 44 770 rev./min at 25°C in a Spinco model E analytical ultracentrifuge. A standard cell with a Kel-F centrepiece was used in an AN-D rotor. Photographs of E_{260} taken on Ilford commercial ortho film were scanned with a Joyce-Loebel microdensitometer (50 µm slit width). Approximate values for the density of components relative to the marker DNA were calculated using the method of Schildkraut *et al.* (1962).

Melting temperatures

The DNA solution that had been dialysed for 18 h against 0.014M-NaCl–0.0015M-trisodium citrate buffer, pH 7.0, was placed in a stoppered cuvette in a Pye-Unicam electrically-heated cell housing in a Unicam SP.800 spectrophotometer fitted with a scale-expansion device. A reference cell contained 0.014M-NaCl–0.0015M-trisodium citrate buffer, pH 7.0. E_{260} of the DNA solution at 25°C was determined. The cells were then heated at 0.5°C/min and the extinction at different temperatures was recorded. Temperatures of the DNA solution were monitored with a thermocouple (W. G. Pye & Co., Cambridge, U.K.). The E_{260} values were corrected for solvent expansion (Mandel & Marmur, 1968), and the corrected E_{260} divided by E_{260} at 25°C was plotted against temperature.

Treatment of rats with retrorsine

Male Porton-Wistar rats (130–150 g) were dosed intraperitoneally with retrorsine hydrochloride (30 mg/kg) in iso-osmotic saline. The animals were killed after 30 min or 2 h by a blow on the head, and exsanguinated. The concentration of pyrrolic metabolites was determined in 0.5 g portions of the liver (Mattocks & White, 1970), and the remainder was transferred to ice-cold 0.32M-sucrose for DNA extraction.

Results

Melting temperatures

The effects of pretreating *E. coli* DNA with various concentrations of monocrotaline pyrrole (compound I) (619 µM–3.1 mM) on the cooling curves are shown in Fig. 1. None of the pyrrolic derivatives tested appreciably affected the shape of the melting curve as the DNA was heated. For clarity, except for the control DNA, the heating part of each curve of DNA pretreated with monocrotaline pyrrole has been omitted. Compared with the control DNA, even the lowest concentration of monocrotaline pyrrole (619 µM) had a detectable effect on renaturation. A

Fig. 1. Thermal-denaturation profiles of *E. coli* DNA

Solutions of DNA in 0.014M-NaCl–0.0015M-trisodium citrate buffer, pH 7.0, were heated or cooled at approx. 0.5°C/min. The E_{260} at different temperatures ($E_{260} t$), corrected for solvent expansion, divided by E_{260} at 25°C ($E_{260} 25$) are plotted against temperature. ○, Heating curve, control DNA; △, cooling curve, control DNA; □, cooling curve of DNA pretreated with 619 μM-monocrotaline pyrrole; ●, cooling curve of DNA pretreated with 1.24mM-monocrotaline pyrrole; ▲, cooling curve of DNA pretreated with 3.10mM-monocrotaline pyrrole.

Fig. 2. Thermal-denaturation profiles of rat liver DNA

Solutions of DNA in 0.014M-NaCl–0.0015M-trisodium citrate buffer, pH 7.0, were heated or cooled at approx. 0.5°C/min. The E_{260} at different temperatures ($E_{260} t$), corrected for solvent expansion, divided by E_{260} at 25°C ($E_{260} 25$) are plotted against temperature. ○, Heating curve, control DNA; △, cooling curve, control DNA; □, cooling curve of DNA pretreated with 619 μM-monocrotaline pyrrole; ●, cooling curve of DNA pretreated with 1.24mM-monocrotaline pyrrole; ▲, cooling curve of DNA pretreated with 3.10mM-monocrotaline pyrrole.

similar pattern was seen for rat liver DNA, although there was a greater degree of renaturation for an equivalent concentration of pyrrolic derivative (Fig. 2).

Equilibrium-density-gradient centrifugation

Alkaline caesium chloride density-gradient centrifugation was done to obtain a better separation of native DNA from the heat-denatured component (Vinograd *et al.*, 1963). *E. coli* DNA treated with monocrotaline pyrrole and then subjected to density-gradient centrifugation showed only a single component equilibrating at a position (relative density 1.71) equivalent to that of native DNA. *E. coli* DNA treated with monocrotaline pyrrole, when heated to 100°C and quenched in ice showed, in addition to the denatured component (relative density 1.75), a con-

stituent equilibrating at a position equivalent to that of native *E. coli* DNA (relative density 1.71) (Figs. 3a, 3b and 3c).

The degree of renaturation was dependent on the concentration of monocrotaline used (Figs. 3c, 3d and 3e). A similar degree of renaturation was obtained whether the DNA was precipitated after 1 or 15 min of exposure to 1.24mM-monocrotaline pyrrole. Changing the tris–HCl buffer for phosphate or Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl]ethanesulphonic acid] had little effect. Renaturation after exposure to monocrotaline pyrrole in 0.04M-sodium acetate–acetic acid buffer, pH 5.5, or 0.04M-tris–HCl buffer, pH 9.0, was as effective as in tris–HCl buffer, pH 7.4.

In one experiment, monocrotaline pyrrole (1.24mM) was added to 0.04M-tris–HCl buffer, pH 7.4, and left for 5 min at 20°C. The *E. coli* DNA sample was then added and the mixture left for a further 5 min. DNA pretreated in this way showed no

Fig. 3. Microdensitometer tracings of equilibrium-density-gradient-centrifugation runs of *E. coli* DNA in alkaline CsCl

DNA samples were centrifuged at 44 770 rev./min for 22 h at 25°C in an AN-D rotor of a Spinco model E analytical ultracentrifuge in CsCl, pH 10.8. Photographs of E_{260} were scanned in a Joyce-Loebel recording microdensitometer. The marker in all traces is *Cl. perfringens* DNA (relative density 1.69). (a) Control *E. coli* DNA (relative density 1.71). (b) Heat-

evidence of renaturation after heat treatment over that of untreated controls.

Retrorsine pyrrole (compound II) behaved in the same way as monocrotaline pyrrole in bringing about the renaturation of *E. coli* DNA after heat treatment (Fig. 4a). Several synthetic hydroxymethylpyrrole derivatives (Mattocks, 1971) were also tested. The monoester, *N*-methyl-2-hydroxymethylpyrrole-*N'*-ethyl carbamate (compound VI) at a concentration of 1.24 mM was ineffective in bringing about renaturation of *E. coli* DNA after heat treatment. The dicarbamate ester (compound IV) at the same concentration, was as effective as monocrotaline pyrrole. The diacetoxy ester (compound V) caused relatively more DNA renaturation (Figs. 4b and 4c).

Melting temperature experiments had indicated that exposure to monocrotaline pyrrole caused rat liver DNA to renature to a greater extent than *E. coli* DNA. Rat liver DNA was exposed to 1.24 mM-monocrotaline pyrrole and was subsequently heat treated. Equilibrium-density-gradient centrifugation showed that under these conditions renaturation was greater than for *E. coli* DNA treated in a similar manner (Fig. 5c).

Treatment of rats with retrorsine

Rats were dosed intraperitoneally with retrorsine (30 mg/kg) and killed 30 min or 2 h later. These times were chosen as 30 min after dosing the highest concentration of pyrrolic metabolites in the liver has been reached, whereas after 2 h a steady concentration plateau of pyrroles exists (A. R. Mattocks, unpublished work). DNA was isolated, but in neither instance did the sample show a significant degree of renaturation after heat treatment, as judged by equilibrium-density-gradient centrifugation (Fig. 5d).

Discussion

Dihydropyrrolizines prepared chemically from the parent alkaloids react with alcohols, amines and nucleic acids (Mattocks, 1969; Black & Jago, 1970; Culvenor *et al.*, 1970).

E. coli DNA, after exposure to monocrotaline pyrrole and after being heated to well above the melting temperature, renatures either on slow cooling, as judged by the decrease in E_{260} , or on quenching in ice, as shown by subsequent equilibrium-density-gradient-centrifugation techniques. Both of these

denatured DNA (relative density 1.75). (c) DNA pretreated with 619 μ M-monocrotaline pyrrole and heat denatured. (d) DNA pretreated with 1.24 mM-monocrotaline pyrrole and heat denatured. (e) DNA pretreated with 3.10 mM-monocrotaline pyrrole and heat denatured.

Fig. 4. Microdensitometer tracing of equilibrium-density-gradient-centrifugation runs of *E. coli* DNA in alkaline CsCl

DNA samples were centrifuged at 44770 rev./min for 22 h at 25°C in an AN-D rotor of a Spinco model E analytical ultracentrifuge in CsCl, pH 10.8. Photographs of E_{260} were scanned in a Joyce-Loebel recording microdensitometer. The marker in all traces is *Cl. perfringens* DNA (relative density 1.69). (a) DNA pretreated with 1.24 mM-retrorsine pyrrole and heat denatured. (b) DNA pretreated with 1.24 mM-1-methyl-2,3-bis-hydroxymethylpyrrole di-(*N*-ethyl)carbamate and heat denatured. (c) DNA pretreated with 1.24 mM-1-methyl-2,3-bis-acetoxymethylpyrrole and heat denatured.

experiments suggest that the pyrrole is covalently cross-linking the DNA strands. Although the bi-functional alkylating capacity of these pyrroles has

Fig. 5. Microdensitometer tracing of equilibrium-density-gradient-centrifugation runs of rat liver DNA in alkaline CsCl

DNA samples were centrifuged at 44770 rev./min for 22 h at 25°C in an AN-D rotor of a Spinco model E analytical ultracentrifuge in CsCl, pH 10.8. Photographs of E_{260} were scanned in a Joyce-Loebel recording microdensitometer. The marker in all traces is alkaline-denatured *M. lysodeiticus* DNA (relative density 1.77). (a) Heat-denatured DNA (relative density 1.74). (b) Control DNA (relative density 1.70). (c) DNA pretreated with 1.24 mM-monocrotaline pyrrole and heat-denatured. (d) DNA heat denatured, taken from rats killed 2 h after intraperitoneal dose of retrorsine (30 mg/kg). A similar pattern was observed in DNA isolated from rats killed 30 min after dosing.

been suggested on theoretical grounds (Mattocks, 1968), this is the first time that such a reaction has been demonstrated.

The half-life of retrorsine pyrrole in aqueous solution is extremely short. After addition to tris-HCl buffer at pH 7.2, its initial high reactivity falls to a much lower level within 1 min, after which there is little further change during 30 min (Mattocks, 1969). Monocrotaline pyrrole and other dihydropyrrolizine esters behave in a similar way. The initial rapid change in reactivity probably corresponds with hydrolysis to hydroxy derivatives, such as dehydroretronecine (compound III), which are less reactive towards nucleophiles. Some reaction with the buffer may also occur.

Possibly because of such competing reactions, interaction with DNA was observed only at relatively high pyrrole concentrations ($619 \mu\text{M}$ – 3.1 mM). This represents up to 1 mol of pyrrole/mol of DNA phosphorus. The actual degree of cross-linkage in each DNA molecule is probably very low since (a) renaturation was never complete after heat treatment and (b) the pyrrole-treated DNA that had not been heated was of a similar density to native DNA on equilibrium-density-gradient centrifugation. If a large number of pyrrolic residues were covalently linked to each DNA molecule this would presumably be reflected by a change in the density of the DNA that could be detected by density-gradient centrifugation.

The degree of renaturation of heat-denatured *E. coli* DNA after pretreatment with monocrotaline pyrrole was the same whether the DNA was initially exposed to the pyrrole for 1 or 15 min. No effect was seen if the pyrrole was added to aqueous buffer for 5 min before adding the DNA. These results indicate that the cross-linking of the DNA is caused by the short-lived esters during the first minute and not by the less reactive hydroxy derivatives.

It is not known which groups of the DNA are alkylated by the pyrroles. Brookes & Lawley (1961) have shown that with many alkylating agents, e.g. nitrogen mustard [di-(2-chloroethyl)methylamine] or methyl methanesulphonate, the N-7 position of the guanine residues was the site most open to attack. It has been suggested (Mattocks, 1969; Culvenor *et al.*, 1969) that metabolically activated mitomycin, which has a reactive carbamate ester, might act in a similar manner to the dihydropyrrolizine diesters. However, the cross-linking of DNA by mitomycin apparently does not involve the N-7 position of guanine (Tomasz, 1970).

The $(A+T)/(G+C)$ ratio for rat liver DNA (1.37) is slightly higher than for *E. coli* DNA (1.01) (Chargaff, 1955). The greater apparent degree of cross-linking of rat liver DNA by the pyrrolic derivatives might be due to differences in base composition or to other factors, such as the degree of protein contamination of the DNA preparations.

Although retrorsine pyrrole effectively cross-linked rat liver DNA *in vitro*, this effect could not be

detected *in vivo* when rats were given an intraperitoneal injection of the alkaloid retrorsine. The dose used (30 mg/kg) was slightly less than the acute LD_{50} (35 mg/kg) (Schoental, 1968). Pyrrolic metabolites from retrorsine are formed in the microsomal fraction of the liver (Mattocks & White, 1971). To react with DNA the reactive metabolites would have to diffuse into the nucleus without interacting with water or tissue nucleophiles and with the techniques described in the present paper, the proportion of DNA cross-linked would have to be quite high for the effect to be demonstrable.

In conclusion, we have shown that in common with a number of other cytotoxic agents such as the mitomycins and the nitrogen mustards, the reactive pyrrolic derivatives of the pyrrolizidine alkaloids can cause cross-linking of DNA *in vitro*. It remains to be established whether this cross-linkage of DNA is involved in any of these cytotoxic effects.

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