Interaction of Dehydroheliotridine, a Metabolite of Heliotridine-Based Pyrrolizidine Alkaloids, with Native and Heat-Denatured Deoxyribonucleic Acid *in vitro*

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Dehydroheliotridine, a major pyrrolic metabolite of heliotridine-based pyrrolizidine alkaloids, interacted with both native and heat-denatured DNA *in vitro* under mildly acid conditions to form a soluble non-diffusible complex as shown by spectral data. The complex formed with heat-denatured DNA was more resistant than heat-denatured DNA alone to the combined action of deoxyribonuclease and phosphodiesterase. Analysis of the resistant fractions suggested the presence of at least one base not obtained from DNA alone.

Exposure to certain pyrrolizidine alkaloids produces a latent long-lasting mitotic inhibition in liver parenchymal cells (Peterson, 1965; Downing & Peterson, 1968). This inhibition, which may remain latent for up to 3 months after a single administration of alkaloid (Peterson, 1965) is only expressed after a stimulus for growth or regeneration (Jago, 1969). The nature of the biochemical event that results in the mitotic inhibition is not yet known. The relative permanency of the effect suggests the possibility of interaction of the alkaloid or of one of its metabolites with DNA or of some other interference with specific aspects of DNA expression.

Dehydroheliotridine (7-hydroxy-1-hydroxy-methyl-dihydro-5H-pyrrolizidine) has been isolated and identified as the major pyrrolic metabolite produced *in vitro* by rat liver microsomes from the alkaloids lasiocarpine and heliotrine (Culvenor, Downing, Edgar & Jago, 1969), and it has also been recovered from the urine of rats after intraperitoneal administration of these alkaloids (Culvenor *et al.* 1969). As a single administration of dehydroheliotridine to young rats produces a striking though temporary arrest of growth, loss of hair and abnormalities in the teeth (M. V. Jago, unpublished work), this compound was considered as a possible 'active metabolite' and its reactivity with DNA was examined. The present report presents evidence that dehydroheliotridine interacts with DNA *in vitro*. The complex formed was studied by using enzymic and chromatographic methods.

MATERIALS AND METHODS

Calf thymus DNA (highly polymerized type I), dAMP, dGMP, dCMP, dTMP, deoxyribonuclease I (once crystallized) from bovine pancreas (deoxyribonuclease oligonucleotidohydrolase, EC 3.1.4.5) and phosphodiesterase type II from *Crotalus adamanteus* (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. A pure crystalline sample of dehydroheliotridine was generously supplied by Dr C. C. J. Culvenor, Commonwealth Scientific and Industrial Research Organization, Melbourne, Vic., Australia. DEAE-cellulose (DE-11) was obtained from Whatman (W. and R. Balston Ltd., Maidstone, Kent, U.K.).

Interaction of dehydroheliotridine with DNA

Solutions of dehydroheliotridine (80 µg/ml) and DNA (50 µg/ml) were prepared in 25 mm-tris-HCl buffer, pH 7.4 (at 25°C). Equal volumes of each solution were mixed, heated at 100°C for 10 min and rapidly cooled, and any precipitate was removed by centrifugation. The u.v. spectrum (230-290 nm) of the supernatant was measured before and after dialysis against 2 × 13 vol. of 25 mm-tris-HCl buffer, pH 7.4, for 48 h at 4°C. Control solutions of dehydroheliotridine or DNA alone mixed with equal volumes of the tris-HCl buffer were treated in an identical manner.

Effect of pH. Solutions of dehydroheliotridine (100 µg/ml) and native DNA (50 µg/ml) were prepared in a series of 13.75 mm-sodium citrate buffers, pH 3 to 6.4 and in 25 mm-tris-HCl buffer, pH 7.4. Equal volumes of the dehydroheliotridine and the DNA solutions in the same buffer were mixed and incubated at 25°C for 2 h. Any precipitate that formed was removed by centrifugation.

The u.v. spectra (230–290 nm) of the supernatants were measured before and after dialysis for 48 h at 4°C against 2×13 vol. of 25 mM-tris-HCl buffer, pH 7.4. Control solutions of dehydroheliotridine or DNA alone mixed with equal volumes of buffer were treated in an identical manner. This experiment was repeated with the dehydroheliotridine and DNA dissolved in 25 mM-KH₂PO₄ adjusted to pH 7.4 with 1M-NaOH.

Effect of temperature. Solutions of dehydroheliotridine (100 µg/ml) and DNA (50 µg/ml) were prepared in 13.75 mM-sodium citrate buffer, pH 5.0. Equal volumes of each were mixed and portions were kept at 0°C, 25°C and 37°C for 4 h. Any precipitate that formed was removed by centrifugation. The supernatants were dialysed against 2×13 vol. of 25 mM-tris-HCl buffer, pH 7.4, for 48 h at 4°C. The amount of complex formed was determined by measurement of the u.v. spectra (230–290 nm) of the non-diffusible material.

Enzymic degradation of dehydroheliotridine-DNA complex

Formation of the complex. Dehydroheliotridine was complexed with DNA by heating solutions containing 500 µg of dehydroheliotridine/ml and 1 mg of DNA/ml in 25 mM-tris-HCl buffer, pH 7.4 at 25°C, for 10 min at 100°C. The solution was rapidly cooled and any precipitate removed by centrifugation (25 000g for 5 min) in a Sorvall RC2 centrifuge. A solution of the complex prepared in this manner was used in all subsequent studies.

Degradation by deoxyribonuclease. The relative rates of degradation by deoxyribonuclease of DNA and of the DNA-dehydroheliotridine complex were determined by measuring the E_{260} of the released acid-soluble material (Kunitz, 1950; Brooks & Klammer, 1968). Solutions of the dehydroheliotridine-DNA complex and of DNA alone were dialysed for 48 h at 0–4°C against 25 mM-tris-HCl buffer, pH 7.4, before use in this test. Solutions containing (final concentrations) 25 mM-tris-HCl buffer, pH 7.4, 20 mM-MgCl₂, DNA (50 µg/ml) alone or dehydroheliotridine-DNA complex (DNA, 50 µg/ml, plus dehydroheliotridine, 25 µg/ml) or DNA (50 µg/ml) with uncomplexed dehydroheliotridine (25 µg/ml) were incubated with deoxyribonuclease (5 µg/ml) at 25°C. At zero time and 1, 4, 7 and 10 min after addition of the enzyme, 1 ml samples were withdrawn and rapidly mixed with 1.0 ml of ice-cold M-perchloric acid. After the mixture had stood for 30 min in ice the precipitate was removed by centrifugation and the E_{260} of the supernatants was measured.

Degradation by deoxyribonuclease and phosphodiesterase. Solutions containing (final concentrations) 25 mM-tris-HCl buffer, pH 7.4, 20 mM-MgCl₂ and DNA (1 mg/ml) or dehydroheliotridine-DNA complex (DNA, 1 mg/ml, plus dehydroheliotridine, 500 µg/ml) or DNA (1 mg/ml) and uncomplexed dehydroheliotridine (500 µg/ml) were incubated with deoxyribonuclease (10 µg/ml) and phosphodiesterase (5 µg/ml) at 37°C for 21 h. At $\frac{1}{2}$, 1, 2, 4 and 21 h, 40 µl samples of each of the reaction mixtures were withdrawn and analysed by paper chromatography (solvent 1).

Chromatographic analysis of enzymic hydrolysates. Solutions containing 50 mg of DNA, either alone or complexed with 25 mg of dehydroheliotridine, were incubated with deoxyribonuclease and phosphodiesterase

under the conditions described above for 21 h. The reaction mixtures were deproteinized by mechanical shaking for 5 min with an equal volume of chloroform-3-methylbutan-1-ol (24:1, v/v). After centrifuging, the aqueous phase was carefully withdrawn and the dissolved chloroform was removed by a stream of N₂. The enzymic hydrolysates were diluted to 200 ml with 0.01 M-lithium acetate buffer, pH 5.3, and applied to a DEAE-cellulose column (1.5 cm×25 cm) pre-equilibrated with the same buffer. The column was washed with 100 ml of 0.01 M-lithium acetate buffer, pH 5.3, and a linear gradient of LiCl (0–0.4 M in 0.01 M-lithium acetate buffer, pH 5.3, total volume 1.5 litres) was applied to the column. After the gradient elution the column was washed with 1.0 M-LiCl in 0.01 M-lithium acetate buffer, pH 5.3. The column eluate was monitored at 254 nm with a LKB Uvicord and 8 ml fractions were collected. The fractions constituting the individual oligonucleotide peaks were combined, diluted with 2 vol. of water and applied to a DEAE-cellulose column (1 cm×5 cm). Samples of individual oligonucleotide peaks were taken before dilution for phosphorus analysis. The column was washed successively with 100 ml of water and 100 ml of 0.01 M-NH₄HCO₃. The oligonucleotide fraction was removed quantitatively with approx. 10–15 ml of 2 M-NH₄HCO₃. The NH₄HCO₃ was removed by successive evaporation of the solution under vacuum at 37°C in a rotary evaporator. The residues were taken up in a small volume of water and portions were analysed by two-dimensional chromatography (solvents 3 and 4). All the individual spots were eluted with water and their u.v. spectra (230–290 nm) recorded. The eluates were evaporated to dryness. Base analyses of the total fraction and of individual eluted spots were performed by formic acid hydrolysis followed by paper chromatography of the hydrolysate with solvent 2 (Wyatt, 1955).

Paper chromatography

The solvents used were: solvent 1, 95% (v/v) ethanol-1 M-ammonium acetate, pH 7.5 (5:2, v/v); solvent 2, propan-2-ol-conc. HCl-water (170:40:39, by vol.); solvent 3, propan-2-ol-water (7:3, v/v) plus 0.35 ml of ammonia (sp.gr. 0.88)/l tank volume; solvent 4, 2-methylpropionic acid-0.5 M-ammonia (5:3, v/v). Whatman no. 1 paper was used throughout. All chromatograms were developed in a descending manner. For two-dimensional chromatograms solvent 3 was used for the first dimension (72 h) and solvent 4 for the second dimension (24 h).

Phosphorus was determined by the method of Gomori (1941–42). Chemical assay of pyrrolic compounds was carried out by the method of Mattocks (1967, 1968).

RESULTS AND DISCUSSION

Dehydroheliotridine, a white crystalline compound, readily dissolved in water to give a clear colourless solution that absorbed strongly in the ultraviolet region at wavelengths below 250 nm. Although there was no E_{max} at 230 nm, the E_{230} was proportional to the concentration up to at least 50 µg/ml.

When a solution of dehydroheliotridine in tris-HCl buffer, pH 7.4 at 25°C, was heated at 100°C,

Fig. 1. Interaction of dehydroheliotridine with DNA. Dehydroheliotridine and DNA were complexed by heating together in tris-HCl buffer. For experimental details see the Materials and Methods section. O, Dehydroheliotridine and DNA before dialysis; ●, dehydroheliotridine and DNA after dialysis; —, summation of the spectra of dehydroheliotridine and DNA after heating and centrifuging separately; ▲, denatured DNA; □, dehydroheliotridine heated and centrifuged; ■, dehydroheliotridine heated, centrifuged and dialysed.

a fine precipitate formed, which appeared red-brown on collection by centrifugation. The supernatant was colourless and still contained most of the dehydroheliotridine as determined by u.v. spectrum and by chemical assay. However, when a solution containing both dehydroheliotridine and DNA was heated at 100°C, the solution turned a salmon-pink colour but no precipitate was formed. The u.v. spectrum of this solution was different from that of the sum of the spectra of the dehydroheliotridine and the denatured DNA (Fig. 1). Dialysis of the supernatant of the heated dehydroheliotridine solution led to complete loss of the remaining dehydroheliotridine. However, dialysis for up to 1 week of the heated mixture of dehydroheliotridine and DNA did not remove all of the dehydroheliotridine; about one-third was not diffusible. The non-diffusible material was salmon-pink in colour and its u.v. spectrum was different from that of DNA. Thus it appeared that dehydroheliotridine and DNA had interacted with the formation of a complex. When the solution containing both dehydroheliotridine

and DNA in phosphate buffer, pH 7.4, was heated, no change in the colour of the solution was observed. After dialysis, the u.v. spectrum (230–290nm) of the mixture was not different from that of DNA alone. It therefore seemed likely that the interaction was caused by the drop in pH produced by raising the temperature of the tris-HCl buffer (see Datta & Grzybowski, 1961).

The effect of pH on the interaction between dehydroheliotridine and native DNA was studied over the range pH 3–7.4. At pH 4.5 and below, all the DNA (as determined by E_{260}) was co-precipitated with dehydroheliotridine as a red-brown precipitate. Dialysis of the supernatants led to the complete loss of all the remaining dehydroheliotridine (Fig. 2). Over the range pH 4.8–5.2 the reaction mixture turned a salmon-pink colour and a small amount of fine precipitate was formed. As shown by the u.v. spectrum (Fig. 2), a considerable amount of the dehydroheliotridine was non-diffusible, presumably bound to the DNA in the form of a complex. With a further increase of pH to 5.7 the amount of bound dehydroheliotridine was decreased and the amount of diffusible dehydroheliotridine increased. No bound dehydroheliotridine was detected at pH 6.4 or 7.4. Dehydroheliotridine alone was incubated under identical conditions over the same pH range. At pH 5 and below, some dehydroheliotridine was precipitated as a red-brown polymer. The supernatant obtained after centrifuging was colourless and the unprecipitated dehydroheliotridine diffusible. Above pH 5.3 no precipitate formed, the solution remained colourless and the dehydroheliotridine was completely diffusible. Thus at slightly acid pH, dehydroheliotridine also interacted with native DNA to form a complex.

The rate of interaction between DNA and dehydroheliotridine at pH 5 was dependent on temperature. After 4 h at 0°C the reaction mixture was still colourless, although a very small amount of complex had formed as shown in Fig. 3. At 25°C the reaction mixture remained clear but was salmon-pink in colour as substantial amounts of complex had formed. At 37°C the DNA was completely co-precipitated with about 40% of the dehydroheliotridine as a red-brown flocculent precipitate. The remaining unbound dehydroheliotridine was diffusible.

The complexing of dehydroheliotridine with heat-denatured DNA markedly decreased the rate of the hydrolysis of DNA to acid-soluble components by deoxyribonuclease (Fig. 4). The presence of uncomplexed dehydroheliotridine also inhibited the enzymic degradation, but to a smaller extent.

The hydrolyses of DNA, of the dehydroheliotridine-DNA complex and of DNA in the presence

Fig. 2. Effects of pH on the interaction between dehydroheliotridine and DNA. Solutions of dehydroheliotridine and DNA in a series of buffers of different pH were incubated at 25°C for 2 h. Any precipitate that formed was removed by centrifugation and the spectra of the supernatants were determined (a) before dialysis and (b) after dialysis. For experimental details see the Materials and Methods section. ○, pH 4.0; ●, pH 4.65; △, pH 5.25; ▲, pH 5.75; □, pH 7.4.

Fig. 3. Effect of temperature on the interaction between dehydroheliotridine and DNA. Dehydroheliotridine and DNA were incubated together at 0°C, 25°C and 37°C at pH 5. Any precipitate that formed was removed by centrifugation. The u.v. spectra of the supernatants were determined after dialysis. For experimental details see the Materials and Methods section. ○, 0°C; ■, 25°C; ▲, 37°C; △, DNA control.

of uncomplexed dehydroheliotridine by the combined action of deoxyribonuclease and snake-venom phosphodiesterase were compared. Analysis by paper chromatography of the products of hydrolysis showed that the DNA alone and the DNA with uncomplexed dehydroheliotridine were completely hydrolysed to the 5'-mononucleotide level during incubation for 21 h. There were no traces of di-, tri- or any larger oligo-nucleotides. The absence of free nucleosides indicated that contaminating phosphatases were not present. The rate of formation of mononucleotides from the dehydroheliotridine-DNA complex was markedly lower than that from DNA alone. After incubation for 21 h a substantial amount of oligonucleotide material was still present in the hydrolysate from the complex.

When the enzymic hydrolysates from DNA and the dehydroheliotridine-DNA complex were applied to DEAE-cellulose columns, all the u.v.-absorbing material was retained. The absorption profiles of the eluates obtained by washing the columns with a linear gradient of lithium chloride are shown in Fig. 5. The elution profile of the DNA control showed that the four 5'-mononucleotides were not separated under the conditions used, separation being dependent on the number of phosphate groups. No further u.v.-absorbing material was eluted from

the DEAE-cellulose until it was washed with m-lithium chloride. The small quantity of material then eluted contained less than 0.05% of the

Fig. 4. Action of deoxyribonuclease on the dehydroheliotridine-DNA complex. The rate of hydrolysis of the complex and of the controls was determined by measuring the E_{260} of the released acid-soluble material. For experimental details see the Materials and Methods section. $\circ$, DNA alone; $\bullet$, DNA with uncomplexed dehydroheliotridine; Δ , DNA complexed with dehydroheliotridine.

original DNA phosphorus. The elution profile of the hydrolysate of the complex showed that, in addition to the four mononucleotides, u.v.-absorbing material corresponding to di-, tri-, tetra- and penta-nucleotides was present. When the DEAE-cellulose was washed with m-lithium chloride, a fraction that was dark brown in colour and that contained about 15% of the original DNA phosphorus was eluted. This fraction, though dark brown in colour, had a u.v. spectrum similar to that of nucleic acid with a maximum at 260 nm and a minimum at 230 nm. However, the E_{260}/E_{230} ratio (1.31:1) was considerably lower than that of the original DNA (E_{260}/E_{230} ratio 2.36:1). Table 1 shows the phosphorus analysis of the different fractions.

The desalted oligonucleotide peaks were separated into constituent components by two-dimensional paper chromatography. The maximum possible number of natural dinucleotides formed from DNA under the conditions of enzymic digestion used is 16. Fig. 6 shows that the dinucleotide fraction contained 22 individual compounds. The u.v. spectrum of each of the eluted individual spots indicated the presence of nucleic acid bases. Base analysis of spots 2, 7, 8, 10, 11, 12, 14, 19 and 21 showed the presence of not more than two bases per dinucleotide spot. Spots 11 and 12 contained a common unknown component of which the R_F value and the u.v. spectrum were not consistent with those of any of the four bases derived from formic acid hydrolysis of calf thymus DNA. Two-dimensional chromatographic analysis of the trinucleotide fraction gave a complex pattern with at

Fig. 5. Chromatography of the enzymic hydrolysate of the DNA control and of the dehydroheliotridine-DNA complex on a DEAE-cellulose column. The hydrolysate was eluted by a linear gradient of LiCl. For experimental details see the Materials and Methods section. ----, E_{254} of DNA control; —, E_{254} of dehydroheliotridine-DNA complex; ····, LiCl concentration.

Table 1. *Distribution of oligonucleotide fractions released by enzymic hydrolysis of dehydroheliotridine-DNA complex*

For experimental details see the Materials and Methods section.

Fraction no.	Chain length of oligonucleotide fractions	Phosphorus content (%)
1	—	—
2	1	26.2
3	1	21.0
4	2	9.1
5	3	9.4
6	4	6.1
7	5	3.0
8	—	1.8
9	Not known (but larger than 5)	14.7
		91.3

Fig. 6. Two-dimensional chromatogram of the dinucleotide fraction from the DEAE-cellulose column analysis. The desalted dinucleotide fraction was separated into its individual components by two-dimensional chromatography: first dimension, 72 h in solvent 3; second dimension, 24 h in solvent 4.

least 13 individual components. The components of the tetranucleotide fraction and the fraction eluted with *m*-lithium chloride were not separable by two-dimensional paper chromatography. Base analysis of the total tetranucleotide fraction showed the presence of at least seven different components, as shown by both R_F values and u.v. spectra. One of these components gave a brilliant white fluorescence under u.v. light and had a spectrum suggestive of the presence of pyrrolic derivatives. Another of the unknown components had an R_F value and

u.v. spectrum very similar to those of the unknown component derived from the formic acid hydrolysis of the dinucleotide fraction. On formic acid hydrolysis the fraction eluted with *m*-lithium chloride showed the presence of the four natural bases. However, a considerable amount of this fraction was not solubilized by the formic acid hydrolysis.

Dehydroheliotridine and other pyrrolic derivatives have been found to form red polymers spontaneously under acid conditions (Mattocks, 1968; Culvenor *et al.* 1969). In the present work it was found that, under mildly acid conditions, the presence of either native or heat-denatured DNA in a solution prevented the formation and precipitation of the dehydroheliotridine polymer. The spectral evidence suggested that the dehydroheliotridine had interacted with the DNA to form a soluble complex. Although the interaction was dependent on pH, the nature of this pH-dependence is not clear. It may be related to the protonation of the hydroxyl groups of the dehydroheliotridine that becomes significant at about pH 5. As the protonated hydroxyl is a good 'leaving' group, dehydroheliotridine is thus converted into a strong alkylating agent capable of reacting by a mechanism involving a free carbonium ion (Culvenor, Edgar, Smith & Tweeddale, 1970). With further decrease in pH the DNA was co-precipitated with the dehydroheliotridine, possibly by the stacking of more dehydroheliotridine molecules on to those already bound to the DNA strands and also by the formation of cross-links between stacks of dehydroheliotridine on adjacent strands of DNA. The rate of co-precipitation was a function of the concentration of dehydroheliotridine, the pH and the temperature. Extremely low concentrations of dehydroheliotridine (1–5 $\mu\text{g/ml}$) did not co-precipitate DNA but produced a gel (D. N. Black & M. V. Jago, unpublished work), suggesting the formation of interstrand cross-links.

The soluble dehydroheliotridine-DNA complex was more resistant than DNA to enzymic hydrolysis by deoxyribonuclease and phosphodiesterase. Further evidence for the interaction between the two compounds was given by the presence of an unnatural base in the non-digested fractions. The fraction that was eluted from DEAE-cellulose by *m*-lithium chloride, containing 15% of the original phosphorus, had a u.v. spectrum similar to that of DNA. However, the dark-brown colour and the E_{260}/E_{230} ratio of this fraction suggested the presence of complexed derivatives of dehydroheliotridine.

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