A T4-Bacteriophage Reversion Test for the Naturally Occurring Mutagen Present in Bracken Fern (*Pteridium aquilinum*)

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Previous work had shown that certain bracken extracts caused dominant lethal mutations in *Drosophila* and gave positive tests in a mammalian mutagen test (Evans, Barber, Jones & Leach, 1968, 1969). These fractions also contained the agent responsible for bracken poisoning of cattle and carcinogenic activity in small animals (Evans & Mason, 1965). To facilitate isolation of the active principle(s) a more rapid and sensitive test was desirable.

Mutant T4 bacteriophages produced by certain classes of mutagens can be induced to revert to the wild-type by the application of some other mutagenic agents (Freese, 1959). Thus, by using a T4 rII mutant bacteriophage, which will not lyse an *Escherichia coli* strain with a λ prophage, it might be possible to make it revert to the wild-type by using an unknown mutagen. The revertant r⁺ genotype could then be detected in the presence of the original rII mutant by using *E. coli* KB (λ) as indicator.

Nutrient agar plates were inoculated by spreading 3ml of sloppy nutrient agar (Oxoid; 1.3g/100ml) containing 0.1ml of a suspension of *E. coli* K (overnight growth, aerated in nutrient broth at 37°C), 0.1ml of a suspension of *E. coli* B (similarly grown, but diluted 1:10 with nutrient broth) and 0.1ml of stock T4 rII bacteriophage mutant (titre approx. 4×10^7 /ml); a control plate is inoculated by omitting the bacteriophage. The plate was divided into two halves and a hole (4mm diam.) gouged in each. An aqueous solution of mutagen or bracken extract (0.1ml) was placed in the holes; the plates were then left (for 2h at 4°C) to allow diffusion of the mutagen while the growth of bacteria and bacteriophage is inhibited. They were incubated overnight (37°C) and inspected. Results and interpretations can be divided into three categories.

(i) Substances that do not cause reversion of the rII mutant: bacterial growth extends evenly across the plate, and spontaneous revertants appear as a number of small plaques spread evenly across the surface.

(ii) Mutagens that cause reversion: a lysis zone is seen around the hole, the area being dependent on the nature of the mutagen and its concentration.

(iii) Mutagens that are also bactericidal or bacteriostatic: the clear area in the vicinity of the hole is caused by bacterial inhibition, as is confirmed by the control plate. Around this area there

sometimes occurs a ring of bacterial growth, possibly because the host cells have mutated to make them bacteriophage-resistant, or the bacteriophages themselves are inhibited in this region. Outside this circle occurs the lysis zone, where the purely mutagenic effect is strongest.

The test gives a positive response with about 1mg of the base-analogue-type mutagens, and with 0.1mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The bracken mutagen, eluted from a single spot on a t.l.c. plate, was active at a concentration of 0.25mg. The method is useful, because of its speed and sensitivity, in following the chemical fractionation of this active principle.

Results obtained with this test and the possible mode of action of the mutagen will be discussed.

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Isolation of an Active Principle from the Bracken Fern that is Mutagenic, Carcinogenic and Lethal to Mice on Intraperitoneal Injection

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The principle(s) in bracken that cause bracken poisoning of cattle was extracted from the dried plant into boiling ethanol (Evans, Evans, Thomas, Watkin & Chamberlain, 1958); thiaminase, which is also present in bracken (Evans, Jones & Evans, 1950), was thus excluded as a factor in the aetiology of the bovine syndrome. Since then, this ethanol extract has been shown to contain a mutagen and a carcinogen; there is considerable evidence indicating that these biological activities are due to the same principle(s). With the aid of a variety of bioassays, (mutagenicity studies using mice and *Drosophila* and the acute and subacute effects following parenteral administration to mice) a toxic chemical has now been isolated and partially characterized by the following procedure.

A hot ethanol concentrate of green dried bracken is poured into excess of cold water and the precipitate discarded. After concentration at low temperature and reduced pressure, the aqueous fraction is treated with charcoal and filtered. The charcoal is eluted with methanol, and the methanol eluate is

added to the aqueous filtrate and concentrated to a syrup *in vacuo*. The concentrate is dissolved in methanol and diluted with excess of ethyl acetate, and the precipitate removed. The solvents are evaporated and the residue is dissolved in methanol and fractionated on a column of Sephadex LH-20 with methanol as eluent. The fractions showing biological activity are pooled and the solvent is removed. The residue is dissolved in water and passed down a column of Amberlite IR-120 cation-exchange resin; the aqueous eluate is evaporated to a pale-yellow viscous residue and dissolved in methanol for chromatography. Chromatography is done on Merck micro-crystalline cellulose plates in butan-1-ol-acetic acid-water (12:3:5, by vol.). After development, the band running at R_F 0.45 is removed, packed into a small column and eluted with methanol. On removal of the solvent a clear colourless gum remains, which has crystallized on occasions to give feathery white crystals, m.p. 110°C.

Mass spectrometry indicates a parent ion of m/e 156, molecular formula $C_7H_8O_4$, and implies the presence of four double bonds or their equivalent. The spectra suggest a furan derivative, and there is evidence for a $-CH_2-CO_2H$ group. The n.m.r. spectrum showed the absence of furan ring protons and of CH_3- or $-CH_2-$ groups that are not subject to a strong downfield shift by other groups. The spectrum consists of a broad hump at 5.5 p.p.m. with two sharper peaks coincident with it; these absorptions are in the olefinic region.

The i.r. spectrum shows the following peaks: 3340 ($-OH$), 2860 (alkyl) and 1690–1700 cm^{-1} ($=CO$). The u.v. spectrum shows only end-absorption at 205–210 nm.

The isolated material is very soluble in water, less so in methanol; solutions have only a limited stability, even at $-20^\circ C$. It appears to polymerize when heated under acid conditions; alkali causes complete loss of activity. There is some evidence that the substance can be formed by very mild hydrolysis of the precipitate from the ethyl acetate fractionation (Leach, 1970). The substance is acidic, and gives a positive reaction for lactones and/or esters. It decolorizes neutral aqueous potassium permanganate, and this reaction has been used for locating the bands on t.l.c. plates; reactions for aldehydes and ketones are negative (Tollens reagent and 3-methylbenzylthiazolone).

Mutagenic and carcinogenic activity are shown by the isolated material; after intraperitoneal injections (10 mg) to mice death occurs within a few hours, with haemorrhages and denudation of the intestinal mucosa. With 2–3-month-old calves fractions are active up to the ethyl acetate supernatant stage; the formidable task of preparing enough of the material isolated by t.l.c. for trial on

the bovine animal has not yet been attempted. Possible structures for the bracken toxin will be discussed.

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Alkaline Reductive Cleavage of 4-*N*-(2-Acetamido - 2 - deoxy - β - D - glucopyranosyl) - L - asparagine

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Treatment of glycoproteins, which contain O-glycosidic linkages to serine and threonine residues, with alkaline sodium borohydride was introduced to obtain reduced oligosaccharides (Schiffman, Kabat & Thompson, 1964) and to examine the amino acid residues forming the point of attachment of the carbohydrate prosthetic groups (Tanaka, Bertolini & Pigman, 1964). Present results suggest that similar procedures might be applied to the preparation of reduced oligosaccharides from glycoproteins or glycopeptides that contain 4-*N*-(2-acetamido - 2 - deoxy - β - D - glucopyranosyl) - L - asparagine (I) as a carbohydrate-peptide linking moiety. Studies of the products formed when compound (I) and 4-*N*-(β -D-glucopyranosyl)-L-asparagine (II) are treated with hot aqueous alkali, both in the presence and in the absence of sodium borohydride, have been carried out.

First-order rate constants for the destruction of compounds (I) and (II) in 0.2M-sodium hydroxide at $100^\circ C$ were 6.44×10^{-3} and $5.97 \times 10^{-3} min^{-1}$ respectively. The rate of release of ammonia under these alkaline conditions from compound (I) followed complex kinetics, with an initial lag phase. Approx. 1.5 mol of ammonia was produced after a time equivalent to five half-lives for destruction of compound (I). U.v.-absorbing material was formed (λ_{max} , 264 nm) with a minimum molar extinction coefficient of 1300, this value being based on the concentration of compound (I). L-Aspartic acid was formed from compounds (I) and (II) with first-order rate constants of about 2.8×10^{-3} and $2.6 \times$