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Porphyrin Accumulation in Sheep Bones Associated with 1,2,4-Trichlorobenzene

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In an earlier report (ROUSSEAUX 1979) details are given of an unusual occurrence of pink fluorescent bones in sheep slaughtered at an abattoir in Portland in the state of Victoria. About 300 carcases were affected. At that time attempts to extract the fluorescent material from the bone were unsuccessful. Although it was suggested that the material may be porphyrin, feeding trials of Phalaris arundinacea and of aminotriazole and ammonium thiocyanate (Weedazole T) failed to reproduce the condition. It was, however, observed that the sheep were watered from a polluted stream.

Earlier this year sheep carcases with bones which fluoresced pink under ultraviolet light were again seen at the Portland abattoir. The sheep were from the same affected flock. This communication describes the identification of the fluorescent pigment and reports on some factors which are associated with the condition.

METHODS

Extraction of the fluorescent pigment

Extraction of the pigment from the bone was achieved by milling roughly broken sections of humerus and scapula in a Spex freezer mill under liquid nitrogen. The finely milled bone (1 g) was suspended in dry methanol (200 mL) and saturated with dry hydrogen chloride. The suspension was allowed to stand at room temperature for 24 hours and was filtered. Examination under long wavelength U.V. light showed that all of the pink fluorescent substance was now dissolved in the methanol. Following dilution of the filtrate with one volume of saturated sodium acetate and one volume of water the pigment was extracted into ether. The residue, after evaporation of the ether to dryness, was subjected to thin-layer chromatography on Merck Kieselgel 60 foil backed plates using the method of SMITH (1975). After hydrolysis of the methyl esters of the pigments to the free acids, electrophoresis was carried out using the method of LOCKWOOD & DAVIES (1961). Protoporphyrin methyl ester and 2,4-(1'-methoxyethyl) deuteroporphyrin were prepared by methods given by FALK (1964). Coproporphyrin III dimethyl ester was from Calbiochem. Isocoproporphyrin methyl ester was isolated from the faeces of rats poisoned with hexachlorobenzene.

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Quantitative analysis of porphyrins in the methanol extract was carried out by scanning the spectrum of the extract from 390 nm to 425 nm and by correcting the absorbance at the maximum for non-porphyrin chromogens using the method of RIMINGTON & SVIENSSON (1950).

Fluorimetric examination of the milled bone was carried out by suspending the bone (1 mg) in ethylene glycol (3 mL) and scanning the excitation and emission spectrum with a Perkin Elmer 3000 spectrofluorimeter.

Extraction and gas chromatography of sheep fat

Fat adhering to the bone samples (5 g) was chopped roughly and extracted by refluxing with cyclohexane (20 mL) for 10 hours. After filtration, the extract was evaporated to dryness and the residue dissolved in n-pentane (5 mL). The solution was applied to a column of Merck activity III alumina (1.5 cm x 5 cm) and eluted with n-pentane. Eight 5 mL fractions were collected. The column was then eluted with ether, four 5 mL fractions being collected, followed by 15 mL of acetone. The fractions were then dissolved in acetone and subjected to gas chromatography using a Pye-Unican 104 gas chromatograph equipped with dual flame ionisation detectors. A similar alumina column was used to test recovery of lindane and the trichlorobenzene isomers. In all cases recovery was better than 90%.

Tissue culture testing of substances which may have been associated with porphyrin accumulation

The embryo chicken liver system of GRANICK (1966) as modified by SASSA & KAPPAS (1977) was used. Williams' medium E from Flow Lab Laboratories was used supplemented with triiodothyronine, insulin and prednisolone phosphate at final concentrations of 1 µg/µL, 1 µg/mL and 0.3 µg/mL. The livers of the chicken embryos were disaggregated using collagenase (Sigma Type I) at a concentration of 0.5 mg/mL in calcium and magnesium free modified Eagles medium. Formation of a monolayer occurred after 20 hours whereupon the medium was changed and the agent to be tested was added in a volume of solvent no greater than 0.1 mL to 3 mL of medium. The monolayers were incubated for a further 24 hours and the cultures analysed for total porphyrin using the fluorimetric method of SINCLAIR & GRANICK (1974). Porphyrins were extracted from the cultures using n-amyl alcohol. After re-extraction into a small volume of 3 M hydrochloric acid, the porphyrins were esterified with methanol 5% sulphuric acid and subjected to thin-layer chromatography.

Suppliers of compounds tested in the tissue culture system and as standards for gas chromatography were as follows: trichlorobenzene isomers were from B.D.H.; lindane was from Lane Agricultural Chemicals; Weedazole T was from Ciba Geigy; 3-amino-1,2,4-triazole and gramine were from Sigma.

Identification of the pigment

Details of the fluorescence spectrum of the milled bone suspension are given in TABLE I. The excitation and emission maxima are compared with those of protoporphyrin and coproporphyrin and show maxima intermediate between these two compounds.

TABLE I

Fluorescence spectra of milled bone suspension compared with the spectra of coproporphyrin and protoporphyrin methyl esters

Solvent	Emission Maxima (nm)		
	Bone Suspension	Coproporphyrin	Protoporphyrin
Ethylene glycol	582, 627 (402)	623 (399)	634 (404)
1N HCl in ethylene glycol	599, 657 (402)	593, 650 (400)	603, 658 (415)
5% zinc acetate in ethylene glycol	582, 627 (401)	578, 626 (402)	590, 640 (417)

Excitation maxima are given in parentheses.

After extraction from the bones, thin-layer chromatography showed the presence of three red fluorescent compounds with R_f values identical with those of protoporphyrin dimethyl ester, 2,4-di(1'-methoxyethyl) deuteroporphyrin dimethyl ester and coproporphyrin tetramethyl ester. Uroporphyrin methyl ester and isocoproporphyrin methyl ester could not be detected. The relative concentrations of the porphyrins were determined by scraping off the bands, eluting the porphyrins with chloroform : methanol (9:1) and measuring the absorbance at the Soret maximum. Protoporphyrin methyl ester and 2,4-di(1'-methoxyethyl) deuteroporphyrin dimethyl ester were treated as one band (see below). The concentrations of the dicarboxylic acid porphyrins and of coproporphyrin in the bone are given in TABLE II.

On electrophoresis the porphyrin carboxylic acids separated into two bands corresponding to the porphyrin dicarboxylic acids and coproporphyrin. No other bands could be seen.

TABLE II

Concentration of porphyrins in sheep bone

Porphyrin	Concentration (picomoles/kg)
Pink fluorescent bone	
Dicarboxylic acid porphyrins	3.55
Coproporphyrin	4.67
Controls	
Dicarboxylic acid porphyrins	0.74 ± 0.23*
Coproporphyrin	not detected

* Value is mean ± S.E.M. for six samples.

Gas chromatographic analysis of sheep bone fat

Other than triglyceride, which had the same retention time as the solvent, only one major peak was observed using the three liquid phases OV-17, DEGS and EGSS-X. This peak was found in the third ether fraction following fractionation of the fat on alumina. Control samples of sheep fat showed no peaks other than triglyceride. Comparison with standard compounds showed the peak to have an identical retention time to that of 1,2,4-trichlorobenzene. Trace amounts of 1,2,3-trichlorobenzene and 1,3,5-trichlorobenzene were also present. Retention times are given in TABLE III.

TABLE III

Gas chromatographic data on components of sheep fat

Liquid Phase	Retention Time (min)		
	Third ether fraction of alumina column	1,2,4-trichlorobenzene	Trichlorobenzene isomers formed by reaction of lindane with KOH
OV17 - 3% on Gas Chrom Q	(2.08)* 2.44	2.44	(2.08) 2.44 (2.78)
DEGS - 6% on Chromosorb W (AW/DMCS)	(0.95) 1.06	1.06	(0.95) 1.06 (1.68)
EGSS-X - 10% on Chromosorb W (AW/DMCS)	(2.66) 3.20	3.20	(2.66) 3.20 (3.88)

* Values in parentheses are minor components.

Columns were glass and were 2.1 m x 4 mm I.D. except for EGSS-X which was stainless steel and 2.8 m x 4 mm I.D.
Carrier gas was nitrogen in all cases at a flow rate of 30 ml/min.
Column temperature was 195°.

The concentration of 1,2,4-trichlorobenzene was determined by the use of appropriate standards and was found to be 120 μ moles/kg.

Tissue culture testing of agents which may be associated with the accumulation of porphyrin

Amongst the agents which have been suggested may be associated with porphyrin accumulation in the sheep bones are aminotriazole and thiocyanate ions (Weedazole T) which had been used to control Typha sp. in the water course used to water the sheep; also, by implication, Phalaris alkaloids, of which the principal alkaloid in P. arundinacea (CULVENOR et al. 1964) is gramine. As the area has a history of infestation with cockchafer beetle larvae. (Aphodius sp.), the recommended agent for control of this pest, hexachlorocyclohexane, and its γ-isomer, lindane, were also implicated. These agents were tested for their ability to cause porphyrin accumulation in tissue culture both in isolation and in combination with the chlorinated hydrocarbons. The results are shown in TABLE IV. In all cases the porphyrin accumulating was protoporphyrin except in the case of lindane where the porphyrin was a mixture of protoporphyrin and coproporphyrin in a ratio of 2:3.

TABLE IV

Effect of agents suspected of causing porphyrin accumulation on chicken embryo liver cells in culture

Agent	Porphyrin accumulation 20 hours after agent addition (p mole/mg protein)
None	32
Ammonium thiocyanate (200 μg/mL)	40
Lindane (5 μg/mL)	270
Lindane (5 μg/mL) plus ammonium thiocyanate (200 μg/mL)	65
Lindane (5 μg/mL) plus o-phenanthroline (20 μg/mL)	60
o-phenanthroline (20 μg/mL)	230

Values are means for six culture dishes. The spread of values in all cases was no greater than 5% of the mean. Agents were added in alcohol solution except for the chlorinated hydrocarbons which were added in acetone. The following agents did not cause significant elevation of porphyrin levels: gramine (100 μg/mL), aminotriazole (200 μg/mL), 1,2,4-trichlorobenzene (10 μg/mL), 2,4,5-trichlorophenol (10 μg/mL).

DISCUSSION

The results show that the fluorescent pigment absorbed on the bones is a mixture of coproporphyrin and dicarboxylic acid porphyrins. Although, after extraction the dicarboxylic acid porphyrins are a mixture of protoporphyrin and 2,4-di(1'-methoxyethyl) deuteroporphyrin, the latter compound is almost certainly formed by the action of methanolic hydrogen chloride on protoporphyrin. This is confirmed by the position of the excitation maxima in the fluorescence spectrum of the bone suspension. The maxima are intermediate between those of coproporphyrin and protoporphyrin. The fluorescence spectrum also suggests that the pigments are present in the bone as the zinc complex.

Most other reported cases of porphyrin accumulation in the bones of mammals other than man have been associated with congenital erythropoietic porphyria (LEVIN 1975). In these cases the porphyrin accumulating is uroporphyrin I. The accumulation of protoporphyrin and coproporphyrin is unusual and is suggestive of a toxic porphyria where an enzymic block has arisen towards the end of the biosynthetic pathway. Although lead poisoning can give rise to a similar pattern of porphyrin accumulation, attempts to detect lead by anodic stripping voltametry in the bones at levels higher than controls were unsuccessful.

The presence of 1,2,4-trichlorobenzene in the fat adhering to the bone samples provides the most satisfactory explanation for porphyrin accumulation. The association of some chlorinated hydrocarbons with porphyria cutanea tarda in man is well known. Although 1,2,4-trichlorobenzene is weakly porphyrogenic in rats (RIMINGTON & ZIEGLER 1963), this compound did not cause porphyrin accumulation in the chicken liver tissue cultures. On the other hand, 1,2,4-trichlorobenzene is a major metabolite of lindane and other hexachlorocyclohexane isomers (GROVER & SIMS 1965). Lindane caused marked elevation of porphyrin levels in the tissue culture system. In this work the porphyrins found to accumulate were protoporphyrin and coproporphyrin rather than uroporphyrin as reported by SINCLAIR & GRANICK (1974). Lindane is widely used in the area both as a spray and in admixture with fertilizers for control of cockchafer larvae. On the other hand no history of use of 1,2,4-trichlorobenzene could be found.

The inhibition of porphyrin accumulation due to lindane by thiocyanate was observed when investigating the possible implication of Weedazole T in porphyrin accumulation. This effect suggests that the porphyrin synthesis inducer is not lindane itself but a metabolite. The active metabolite is not however either 1,2,4-trichlorobenzene or 2,4,5-trichlorophenol. The mode of action of thiocyanate can best be explained by its properties as an iron chelator. This is shown by the action of the iron chelator, o-phenanthroline, which also prevents the lindane associated porphyrin accumulation in tissue culture. In contrast to thio-

cyanate, however, o-phenanthroline alone causes accumulation of protoporphyrin as does the iron chelator, desferrioxamine (SINCLAIR & GRANICK 1974). The most likely explanation for this is that the lipophilic nature of these compounds causes induction of the synthesis of cytochrome P-450 and of δ-aminolevulinic acid synthetase. Thiocyanate is not lipophilic and is unlikely to have an effect on cytochrome P-450 synthesis. The effect of these agents is under further investigation.

Although this work has shown that porphyrin accumulation in the sheep bones is associated with accumulation of 1,2,4-trichlorobenzene in fat, it is likely that both porphyrin and trichlorobenzene accumulation results from exposure to lindane.

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