STUDIES ON SPORIDESMIN METABOLISM IN SHEEP

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SUMMARY

The metabolism of sporidesmin by liver drug metabolizing enzymes was studied using ^{3}H, ^{14}C and ^{35}S labelled sporidesmin. Microsomes, isolated from livers of sheep treated with hexachlorobenzene to induce the liver metabolizing enzymes, were incubated with labelled and unlabelled sporidesmin in the presence of a NADPH generating system. At least 8 different metabolites were found and isolated but to date tentative structures have been assigned only to the monohydroxy and dihydroxy derivates of sporidesmin. A simple assay, which measures the rate of metabolism of ^{35}S-sporidesmin, was used to determine sporidesmin metabolism rates in the 10 000 g supernatant fraction prepared from liver biopsy samples of a number of Romney and Merino sheep.

Results showed a 2- to 3-fold difference in the rate of metabolism of sporidesmin among sheep and a higher metabolism rate in Merino than in Romney sheep. Preliminary results were obtained which showed a correlation of 0.53 between sporidesmin metabolism rates in 17 Romney rams and the average liver damage score of their progeny after sporidesmin dosing. This was close to a theoretical correlation of 0.54 assuming a heritability of resistance of 0.5. From the data it is suggested that it may be possible to use the biochemical assay to select breeding stock for resistance to facial eczema.

INTRODUCTION

Facial eczema is a disease caused by animals ingesting sporidesmin, a toxic compound found in spores of the fungus *Pithomyces chartarum*. The only effective control of the disease to date is by pasture management and by spraying the fungus with a suitable fungicide when spore counts and weather conditions indicate that a facial eczema outbreak is imminent. An alternative approach is to make use of the fact that some animals appear naturally resistant to the disease. Over the last few years investigators at Ruakura have utilized these individual differences by breeding sheep for resistance or susceptibility to the toxic effects of sporidesmin (Campbell *et al.*, 1975). This selection method, however, involves a large-scale progeny testing programme which is slow and only allows a limited number of sires to be selected each year. The present study was undertaken with the view to developing a simple biochemical test for selecting resistant animals and so circumventing the need for progeny testing.

MATERIALS AND METHODS

Tritiated (^{3}H) and carbon 14 (^{14}C) labelled sporidesmin were prepared by growing spores of *Pithomyces chartarum* on a synthetic medium containing ^{3}H- and ^{14}C-tryptophan according to the method of Towers and Wright (1969). Sulphur-35 (^{35}S) labelled sporidesmin was prepared by a similar technique using $Na^{35}SO_4$ as substrate (Brook and Mathews, 1960). The spores were extracted with methanol and then chromatographed on columns of Lipidex-5000 and silica gel using heptane:ether as the eluting solvent. The microsomes used for metabolite identification studies were prepared from livers of two Romney sheep treated with hexachlorobenzene 10 days before slaughter to stimulate production of the hepatic drug metabolizing enzymes. In this experiment ^{3}H- and ^{35}S-sporidesmin and 0.5 g unlabelled sporidesmin were incubated for 30 min at 37° C with microsomes and a NADPH generating system. The incubation mixture was then extracted with ethyl acetate and the crude product chromatographed on Lipidex-5000 using a step-wise elution with hexane: chloroform (Fairclough *et al.*, 1977). The column eluate was monitored for radioactivity and the various peaks containing the ^{3}H or ^{35}S label were rechromatographed on columns of silica gel. Mass spectral data of the purified metabolites were recorded with a Varian CH-5 mass spectrometer using direct probe sample introduction (120-150° C) and an on-line data system. Infrared data were obtained using KBr pellets with a Beckman infrared spectrometer.

Animals used for the biological experiments included 17 Romney rams, 12 Romney ram lambs (sired by rams selected for resistance or susceptibility to sporidesmin dosing) and nine feral sheep of Merino ancestry. The Romney rams were progeny tested by dosing 11 progeny from each sire with a crude sporidesmin extract. The progeny were slaughtered six weeks after dosing and the average liver damage score of the progeny from each sire calculated (Smith *et al.*, 1977). Liver samples were obtained from all animals by biopsy and a 10 000 × g supernatant fraction prepared by homogenizing the liver sample with 4 vol Tris-HCl buffer and then centrifuging. For the enzyme assay ^{35}S-sporidesmin (100 μM) was incubated with the 10 000 g supernatant fraction for 30 min at 37° C. The reaction was then stopped by adding ice cold ethyl-acetate to extract unconverted sporidesmin. The aqueous phase was counted for radioactivity to give the rate of metabolism of sporidesmin in pmol/mg protein/min.

RESULTS AND DISCUSSION

Sporidesmin, labelled with ^{3}H, ^{14}C or ^{35}S was chromatographed to radiochemical purity on columns of Lipidex-5000 and silica gel. Purity was assessed by thin layer chromatography and mass spectroscopy. From a knowledge of the chemical structures of the radioactive substrates, the pathway of biosynthesis of sporidesmin and the fragmentation pattern of the labelled sporidesmin in the mass spectrometer it was possible to deduce the position of each label on the sporidesmin molecule (Fig. 1).

Tracer amounts of ^{3}H- and ^{35}S-sporidesmin were used as an aid in isolating the metabolites produced by incubating sporidesmin with hepatic microsomes and the appropriate co-factors. The elution pattern of the ethyl acetate extracts on Lipidex-5000 columns revealed the presence of at least eight different metabolites (Fig. 2). Attempts have been made to elucidate the structures of the metabolites but to date it has only been possible to assign tentative structures to the monohydroxy (compound H) and the dihydroxy (compound I) derivatives of sporidesmin (Figs. 2 and 3). However, the finding that the two main ethyl acetate extractable metabolites (compounds H and I) do not contain any sulphur atoms is of some importance because Done *et al.* (1961) have shown that the cytotoxicity of sporidesmin in cell culture is associated with an intact epidithiodioxoepipiperazine ring. In fact, only small alterations to the disulphide bridge such as those seen in sporidesmin-D where the -S-S- bridge is open (Jamieson *et al.*, 1969) results in a marked reduction in biological activity (Done *et al.*, 1961). It is likely, therefore, that the toxicity of compounds H and I would be low compared with sporidesmin. This proposal is compatible with recent observations of S. D. Aust (pers. comm.) who showed that the ether extractable metabolites formed from sporidesmin by hepatic microsomes gave only a small inflammatory response in rats. It

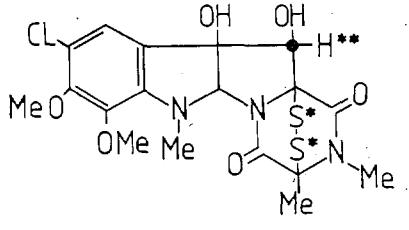

FIG. 1: *Structure of sporidesmin. The positions of each label are shown. Carbon-14* (●), *sulphur-35* (*), *tritium* (**).

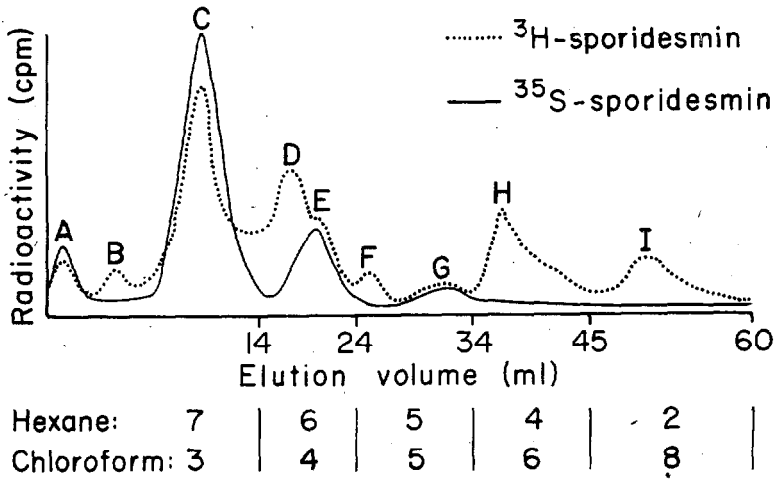

FIG. 2: *Lipidex-5000 chromatography of metabolites produced by incubating sporidesmin with hepatic microsomes from sheep. The various compounds are labelled A to I, with sporidesmin corresponding to compond C.*

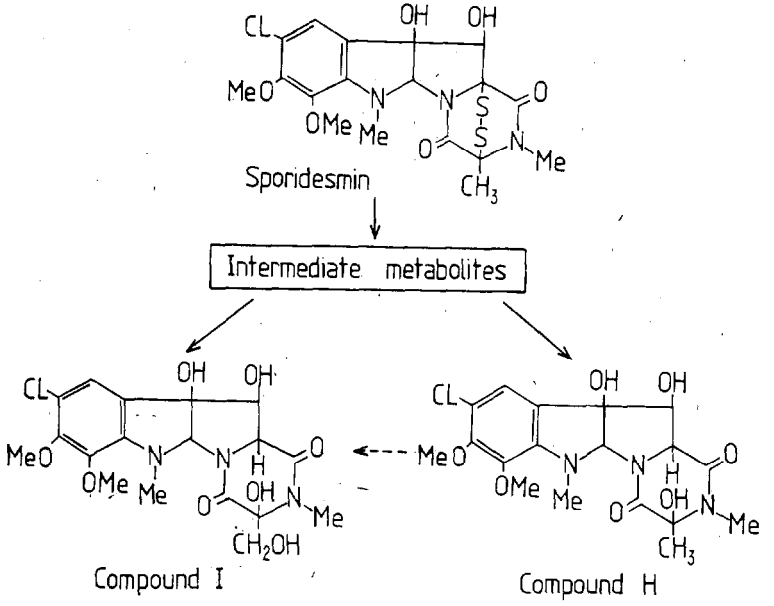

FIG. 3: *Tentative structures of compound I and compound H produced from sporidesmin by hepatic drug metabolizing enzymes in liver.*

would seem reasonable to conclude, therefore, that sporidesmin is detoxified by the drug metabolizing enzymes in liver.

The finding in this study that the sulphur atoms of sporidesmin are converted by hepatic microsomes to mainly water-soluble compounds meant that a simple assay could be established to measure the rate of metabolism of sporidesmin by liver subcellular fractions *in vitro*. The assay, which quantitates the rate of appearance of ^{35}S into the aqueous phase of 10 000 g hepatic supernatant fractions (after ethyl acetate extraction), is simple to perform and is sensitive enough to measure changes in sporidesmin metabolism rate using only 40 μg protein. Such an assay has been used to determine sporidesmin metabolism rates in the 10 000 g supernatant fractions obtained from liver biospies of a number of Romney and Merino sheep. The results depicted in Fig. 4 show a 2- to 3-fold variation in metabolism rate for both Romney and Merino sheep. In addition, the Merino sheep appeared more efficient at metabolizing sporidesmin than the Romney sheep. This observation which confirms earlier work of S. D. Aust (pers. comm.) is interesting because it has been shown that Merino sheep are more resistant to sporidesmin dosing than Romney sheep (B. L. Smith, unpublished). Resistance to facial eczema or sporidesmin dosing can be enhanced in sheep by administering hexachlorobenzene to increase the activity of the

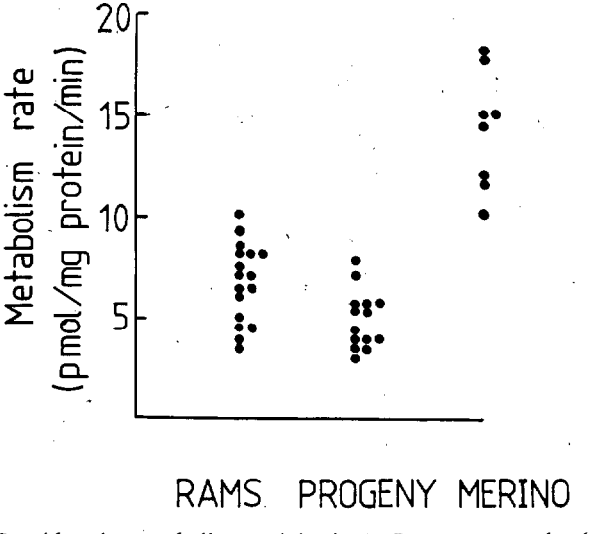

FIG. 4: *Sporidesmin metabolism activity in 17 Romney rams, lamb progeny obtained from selected sires and 9 Merino ewes.*

drug metabolizing enzymes in liver (Mortimer *et al.*, 1978). On the basis of the available evidence the possibility is raised that sheep with more efficient hepatic drug metabolizing enzymes may be naturally resistant to facial eczema. If this is correct, then the metabolism assay could be used to select breeding stock for resistance to facial eczema. This possibility is supported by the results of some preliminary experiments showing a correlation of 0.53 between sporidesmin metabolism rates in 17 Romney sires and the average liver score of their progeny following sporidesmin challenge. This correlation is close to the theoretical value of 0.54 calculated on the basis of a heritability of resistance to facial eczema of 0.5. It is concluded that differences in the rate of metabolism of sporidesmin by hepatic drug metabolizing enzymes may be one of the factors which determine whether sheep are resistant or susceptible to facial eczema.

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