CHEMICAL EXTRACTION AND FRACTIONATION OF THE TOXIN

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IT IS INTENDED only to review the state of this work at the present time, with particular emphasis on the work using fungus cultures as a source of poison. Details of the work on concentrating the poison from grass have been published (White, 1958a). A later paper served as as introduction to a few compounds which were isolated, including the "beaker test" compound (White, 1958b).

The work on grass reached a stage where it was possible to produce as a routine a concentrate containing 1/70,000th of the weight of the grass, and about half the poison of the grass. It took years of slow and invariably frustrating work using a rough guinea pig bio-assay involving feeding for at least four weeks to reach this stage. Most toxic grasses could be processed using this routine, but some have been found where there are difficulties with the last two adsorption chromatographic columns, the presence or absence of certain impurities causing the poison to appear in fractions other than the usual ones.

Using further adsorption and partition chromatographic systems, it was on occasion possible to confine the poison to 1/200,000 to 1/400,000th of the weight of the grass, and it was very easy to destroy the poison. On one occasion the poison was obtained in a fraction from a partition column, representing 1/2,000,000th of the weight of the grass—the fraction was a colourless, glassy solid. It was no comfort to find that fractions of similar weight and physical properties could be obtained from some non-toxic samples; in fact, comparative work has revealed no distinctive chemical or physical features associated with toxicity. The concentration from grass reached a state where it was becoming difficult to do much more fractionation work without using several toxic doses for each experiment. Large scale work on preparing grass concentrates at the 3,000 level has been going on for a year or more at the Plant Chemistry Division, D.S.I.R., and it is pleasing to be able to report that Dr G. W. Butler and J. G. Fraser at Plant Chemistry Division are now able to carry out this processing on a 40 lb basis as well as we can on the 2 lb basis.

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For the meantime efforts to concentrate the poison from grass have been discontinued, but ultimately it must be shown that the poison from pasture and from cultures is the same chemical.

Before disposing of the grass phase of the work mention must be made of the "beaker test" compound. Other papers of this symposium show that the presence of this compound in pasture is often associated with toxicity. It has not been possible to prove that this compound and the poison are related chemically, and there is a much higher concentration of beaker test compound than of poison in grass. There is at the most one in 50,000 of beaker test compound in pasture, and at least one in a few million of poison (in cultures the ratio is at least 50 : 1). It looked as if the beaker test compound was merely an index of the presence of "something" which generally produced the poison, and a suggestion of the nature of this "something" came from early chemical work which showed that the beaker test compound was allied chemically to certain antibiotics from *Streptomyces* and *Fusarium* species.

When J. C. Percival produced scrapings from mower blades at Claudelands and later cultures of *Sporidesmium bakeri,* it was found that the beaker test compound was present in these at higher concentrations than in pasture.

The chemistry of this substance is proceeding. We have several grams of it isolated from cultures and work by Dr R. L. M. Synge leaves little doubt that it is identical with the substance originally obtained from pasture, and the complete chemical structure and even synthesis looks as if it may not be too difficult.

The compound m.p. 149°C, which followed the poison very closely in grass fractionation, has not been found in cultures, and appears to be a genuine ryegrass component.

The chemical studies on the fungus cultures can now be outlined. It was found quite early that a simple ether extract of 1 Roux bottle of culture (200 ml potato-carrot medium, 7 days) gave about the same amount of liver damage as 2 lb of our highly toxic dried grass. Several procedures which worked with grass were then applied to the ether extract, and combined in various ways, and extensive toxicity checks were made on all fractions. A routine was established quite quickly for concentrating the poison in the ether extract to at least 1/20th of the weight of extract with little loss of toxicity. This procedure, which has

been used for batches of up to 100 bottles of culture, is as follows:

(1) Homogenized culture shaken out with 4 lots ether in separating funnels.
(2) Evaporation of ether in cold air blast.
(3) Taking up in a little ether, and insoluble beaker test compound filtered off.
(4) Ether evaporated.
(5) Residue taken up with methanol.
(6) Methanol soluble part diluted to 80 per cent. methanol and shaken with petroleum ether.
(7) Rotary evaporation of methanol to dryness.
(8) Passage through acetic acid de-activated alumina columns using ether.
(9) Cold evaporation of ether.

Many batches of culture have been processed in this way and various modifications designed to facilitate the handling and extraction of 20 l. batches of culture have been made. Unfortunately far too much poison has been lost by using certain of the modifications, such as extracting only the felt part and neglecting the aqueous liquor, and losses in processing occurred when 5-day cultures were used. This was a lesson on the danger of altering any conditions and techniques, some modifications appearing reasonable, without obtaining very clear evidence first that the modifications had no effect on the final result. N. T. Clare has investigated some alternative ways of obtaining a dried ether extract and is mapping out the poison left in side fractions in some of the modifications of the ether extraction procedure. This initial step is the only one which poses a problem in large scale handling and it remains a problem to be solved. Our tentative procedure takes out about 75 per cent. of the poison but is very wasteful of ether. The remaining steps in producing the routine concentrate are on a convenient laboratory scale and can be done in two days.

It is an important point that several batches of culture, when processed by slightly differing initial ether extraction techniques, have given a final concentrate of fairly uniform toxic level on a weight basis; 2 mg when fed by the standard guinea pig feeding technique has given severe liver damage. Referring back to the grass work, the one in 70,000 concentrate of 2 lb grass weighed 13 mg, and usually gave moderately severe damage. Thus, the application to fungus cultures of some of the techniques known

to concentrate the poison from grass, has very easily given a concentrate which is purer than we obtained as a routine from grass, and in terms of grass concentration represents ½ to 1 million.

At this stage appreciation must be expressed of the efforts made by the Soil Bureau chemists, particularly by Dr M. Fieldes, in providing us quickly with ether extract of a lot of fungus. This work showed quite clearly that growing the cultures for 7 days in milk bottles gave the same level of toxicity as obtained from Roux bottles and showed that ether extracts from large volumes (170 bottles) of culture could be successfully evaporated by our methods. When all the Soil Bureau extract was processed here, about 2 g of beaker test compound was obtained, and about 200 mg of final concentrate which gave severe liver damage at the 3 mg level. This is a usable and valuable concentrate, though in terms of numbers of bottles of culture the yield is disappointing—it may have been due to the use of 10-day cultures for some of the fungus.

It remains to mention the application to the routine fungus concentrates of several other methods of concentration developed with grass extracts.

Use of alumina 5 chromatography with benzene and mixed solvents allows at least a doubling of concentration, and this column is being pursued further in an effort to narrow the poison down into a smaller fraction. It may be pointed out that at this stage there is little, if any, colour in concentrates, so for this column traces of a marker have been added (actually carotene) and we have used volume measurement from this band as a guide to the cutting of fractions.

A partition column as used for grass work allowed a doubling of concentration. Dr Synge has used slightly different experimental conditions for this partition column (carbon disulphide/methanol/water) with very encouraging results, the poison appearing in a colourless zone (containing a glassy solid) some distance behind the leading coloured zone containing much of the weight. The small weights now giving high toxicity, and the ease of production of concentrates from fungus, have caused the group at this station to use several toxic doses for each fractionation experiment, but even then it has become necessary to use the microbalance for weighing of fractions, often each a fraction of a milligram. The refinement of these two techniques evolved during

the grass work, either by cutting finer fractions of each, or if necessary by combining the two, should surely allow a much closer approach to the facial eczema poison. The poison occurs in analogous fractions of grass and fungus cultures, providing strong evidence that the poison from both is the same substance. The writer has been saying for a long time that the facial eczema poison is a chemical of extremely potent biological action, and that there is little chance of a practical chemical estimation of the minute quantities which are of importance in pasture samples. It can definitely be said that a total of less than half a milligram of it fed over three weeks followed by a week on non-toxic diet is enough to produce severe liver damage in a guinea pig.

Literature Cited

WHITE, E. P. (1958a): *N.Z. J. Agric. Res., 1:* 433.
——— (1958b): *ibid., 1:* 859.

DISCUSSION

DR A. T. JOHNS: In opening the discussion on Mr White's contribution to the symposium I can best emphasize the difficulties he has had to contend with by repeating a sentence from his paper: "It tooks years of slow and invariably frustrating work using a rough guinea pig bio-assay involving feeding for at least four weeks to reach this stage."

To help Mr White with his purification of the toxic principle we undertook, at the Plant Chemistry Division, to put the extraction procedure on a large scale. A number of modifications were made to the laboratory procedure for large scale work:

(a) The initial extraction is carried out in a Soxhlet type apparatus allowing 20 lb batches of dried grass to be extracted with ether in 16 h. Four extracts are bulked before proceeding to the next step.

(b) Instead of blowing the ether off into the air, the ether extract is concentrated in a cyclone evaporator at room temperature. This equipment handles approximately 30 to 40 l./h with a recovery of 50 per cent. of the ether.

(c) After the treatment of methanol with charcoal, it is washed with petroleum ether by a two stage counter current equipment. (This replaces the use of separating funnels.)

(d) When the washing is completed the methanol is evaporated off in the cyclone evaporator instead of using the Stokes micro-vac pump.

This modified procedure is now working as well as the laboratory scale method.