BIOLOGICAL ASSAYS FOR TOXICITY

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IN THE ABSENCE of any evidence concerning the chemical or physical nature of the facial eczema liver toxin, we are at present forced into the situation of assaying the liver damaging substance by biological methods. This situation presents a number of difficulties. In the first place, animal bio-assays tend to be extremely tedious and time-consuming; again, they are normally most expensive in terms of experimental material and animals; and finally they produce, in general, results of a somewhat variable nature which are often influenced unduly by individual animal differences.

J. B. Swan (pers. comm.) working at Wallaceville in 1943, using dried grass collected in the Gisborne and Wairoa districts which was known to be toxic to sheep, first showed liver damage in guinea pigs, although it was not until 1951 that Evans *et al.* (1957) described the histopathology of the disease in guinea pigs and showed that this species was a suitable one for facial eczema research. Subsequently, Perrin (1957) extended this work, feeding both toxic grass and extracts, and put the guinea pig bio-assay on a more or less quantitative basis.

The two assay procedures in standard use at Ruakura at the present time are, broadly speaking, similar to those recommended by Perrin in his original publication. The feeding procedure used for testing dried grass for toxicity involves a five-week feeding period. Approximately 25 g of grass are fed per day and the weight of the animals at the commencement of the test is around 120 g. At the end of the feeding period the animals are slaughtered and the livers graded arbitrarily by eye to assess the level of toxicity of the test sample. By use of this feeding technique it has been found possible to detect all levels of toxicity from severe samples down to very mild.

The procedure for testing grass extracts for toxicity is to disperse the extract on to non-toxic grass and feed at the rate of 2 lb of the original grass spread over three weeks. Guinea pigs a few days old and weighing about 100 g are used for this purpose. As a routine the animals are left a further seven days on non-toxic grass and slaughtered at the end of the fourth week. This

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has been found necessary in the past to allow the lesions due to low level extracts to develop satisfactorily. A similar procedure is now also in use for testing fungal extracts. While the guinea pig assay in each of its forms has been one of the main factors contributing to the present state of knowledge, it will be obvious that such an assay, in practice, has certain limitations. The feeding periods are long, the assessment of the degree of liver damage is highly subjective, and it is well established that except at the very highest levels of toxicity, the repeatability of results tends to be low. For these various reasons, attempts are being made at Ruakura at the present time to improve the assay procedure.

In seeking to improve upon the present assay methods it is apparent that any alternative procedures must attempt ideally, to reduce:

(a) The time involved.
(b) The amount of toxic material required.
(c) The animal factor.

Such a procedure must also be sufficiently sensitive to pinpoint, for example, losses occurring during chemical isolation of the toxin or to assess varying levels of toxicity such as one might expect to find in successive fractions collected during chromatography.

Current Investigations

The following alternatives are currently under investigation at Ruakura.

Improved Feeding Technique

Attempts are at present being made by N. T. Clare and D. C. Dodd to improve the guinea pig assay by feeding relatively higher levels of toxic extract for shorter periods. The evidence obtained so far in these trials suggests that the feeding time may be able to be reduced to as little as 10 to 14 days providing a sufficiently high daily intake of extract is maintained. The total amount of extract used is the same as that normally fed over 21 days. Such a procedure could, in future, prove useful for a number of purposes such as routine screening of grass extracts, or fungal extracts or cultures, but for chemical work involving lower levels of toxicity it may have a more limited application.

Injection Techniques

Intraportal injection of both rabbits and guinea pigs with toxic extracts of grass and fungus has been shown to produce liver

lesions indistinguishable from those obtained on feeding. This approach has been developed principally in the rabbit with a view to utilizing this species for future biochemical work. The results obtained using this technique are highly repeatable and have the advantage of being evident microscopically in 3 to 6 days. Histologically, the earliest bile duct lesions have been detected in 6 to 12 hours. The method is obviously not one that can be used for routine work involving large numbers of samples, due to the technical difficulty of introducing the toxin, but as an approach to some of the more specialized biochemical problems awaiting solution, the technique is an invaluable one.

A further development in which Ruakura workers have been interested lately is the effect of direct injection of aqueous preparations of toxic extract directly into the gall bladder. Using volumes of between 0.2 and 1.0 ml of material as carrier, the end result of this direct injection technique, using high levels of toxin (equal to 1 l. of fungal culture), is inevitably functional obliteration of the gall bladder, severe generalized oedema and fibrosis of the extra- and intrahepatic biliary system together with gross enlargement of the organ and marked discoloration. The exact mechanics of the situation are not at all clear, but expulsion of the toxic dose into the intestine by way of the common bile duct and re-absorption from the intestine is almost certainly not a factor of any importance. This is the first experimental proof to the knowledge of a direct reaction between toxin and bile duct epithelium, a finding which would tend to substantiate the specificity of the reaction and to discount very largely the role of possible metabolites of the toxin. It may provide also the basis for a refined micro-assay procedure, the possible toxic effects of the facial eczema poison on bile duct epithelial cells in culture being at present under consideration here.

HELA CELL STUDIES

The possibility of an *in vitro* test using tissue culture methods is not new. A series of preliminary trials were conducted last year in collaboration with A. M. Murphy of the Central Laboratory, Auckland Public Hospital, using cultures of the Hela cervical cancer cell. Toxic and non-toxic grass extracts, processed chemically to various stages, fungal extracts and crude fungal preparations were each tested against standard 250,000 cell structures. The results of these experiments indicate that cell growth is readily inhibited and the colonies destroyed within 2 to 4 days of the

introduction of extracts into the culture medium. So-called "non-toxic" grass extracts (that is, extracts prepared from grass which, when fed to guinea pigs was non-toxic) also exhibited some activity, though in general this was of a very much lower order than the corresponding toxic extracts. Further studies along these lines are at present in progress in the hope of developing a rapid *in vitro* micro-assay for toxicity. Present indications are that it may be possible, by means of this technique, to assay an extract from as little as 0.25 g of toxic grass or as little of 0.025 ml of fungal culture.

Investigation of Toxicity in the Mouse

An inflammatory condition of the bile ducts is generally recognized to be the essential feature of facial eczema liver damage in the sheep, guinea pig and rabbit. Recent work at Ruakura has shown this to be true also in the case of the rat. It is noteworthy that there is no convincing evidence in any of these species, with the possible exception of the guinea pig, to suggest a primary effect of the toxin on liver parenchyma.

The mouse, on the other hand, is an interesting exception which does not appear to parallel closely the general pattern of behaviour of these species. In a recent trial toxic extracts were fed at extremely high levels to growing mice over a period of 45 days. During this interval these animals each consumed over five times the amount of extract required to produce severe liver lesions in guinea pigs approximately ten times their weight. Throughout the trial food intake remained normal and good growth was maintained. At slaughter, livers were macroscopically normal and on routine histology no abnormalities were evident in the biliary epithelium. Other animals which had been injected with differing levels of toxic extract and slaughtered at varying intervals thereafter were likewise negative.

On the suggestion of Dr F. A. Denz, of the Toxicology Research Unit of the Medical School, a re-examination of these mice livers, using a frozen section fat-staining technique, has recently been carried out. This approach was developed by Denz and his co-workers as an assay procedure for the active hepatoxic principle which they have successfully isolated from the leaves of the ngaio.

Livers from animals injected with extracts of toxic facial eczema grass, or of fungal culture, exhibit, in many cases, an

acute reaction characterized by areas staining deeply for fat. The distribution of these areas varies but tends, in the main, to be periportal. In chronic cases, such as are obtained on prolonged feeding, the distribution tends to be generalized throughout the liver parenchyma. Examination of affected livers from rabbits, guinea pigs, rats and lambs has failed so far to provide any evidence of fat staining in these species. The exact significance of these observations at the present time is not entirely clear. They would suggest, however, that between the mouse on the one hand, and the rabbit, guinea pig, rat and lamb on the other, there are important basic differences in the site and mode of action of the toxin which require further investigation. The practicability of a mouse assay based on these findings is currently under consideration here. The obvious advantages of such an assay technique, should it prove suitable, in terms of time and materials and animals need not be emphasized.

Literature Cited

EVANS, J. V., MCFARLANE, D., REID, C. S. W., PERRIN, D. D. (1957): *N.Z. J. Sci. Tech. 38A:* 491.

PERRIN, D. D. (1957): *N.Z. J. Sci. Tech. 38A:* 669.