ASPECTS OF THE TOXICOLOGY OF THE
BRACKEN FERN (Pteridium aquilinum)

by

Adetokunbo Olawale Ogunbiyi

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Division of Pharmacology and Toxicology,
Department of Biochemistry,
University of Surrey,
Guildford, Surrey.

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ABSTRACT

Aspects of the toxicology of bracken (Pteridium aquilinum) were studied in sub-acute and chronic studies using male Wistar-albino rats.

In a chronic study, bracken induced ileal adenomas in an 8.5 month period but after 24 months, no tumours were seen in both the quercetin- and shikimate-fed rats.

Tissue ornithine decarboxylase (ODC) levels were enhanced over a 90-day test period when shikimate was fed alone or in conjunction with BBN and MNNG as initiators and with cyclophosphamide, saccharin and lithocholate as promoters, suggesting that shikimate might be a tumour promoter.

Quercetin, was able to enhance ODC levels in conjunction with cyclophosphamide, BBN and MNNG as initiators and with cyclophosphamide, saccharin and lithocholate as promoters. However, it did not enhance tissue ODC levels when administered alone.

Bracken, shikimate and quercetin induced a macrocytic, normochromic, non-regenerative anaemia with thrombocytopenia and granulocytopenia at the termination of the 90-day study.

The bracken-induced ataxia of monogastrics was attributed additionally to nitrite and cyanide, due to the very high levels found in the urine and serum respectively of bracken-fed rats over a 28-day test period.

The high urinary nitrite levels of the bracken-fed rats prompted an investigation into the role of N-nitrosation in bracken-induced carcinogenesis. To this end, the Nitrosation Assay Period (NAP) revealed the presence of nitrosatable entities in the gut of the bracken-, quercetin- and shikimate-fed rats and in the urine of the bracken- and quercetin-fed rats. This suggests that nitrosation, probably via the formation of nitrosamides, might be an important mechanism for the mediation of bracken-induced carcinogenesis.

The plausibility of explaining various aspects of the sub-acute and chronic toxicity of bracken in monogastrics and ruminants on the basis of a "nitrosamide hypothesis" is discussed.
I would like to thank the various persons who assisted in one way or another during the preparation of this thesis, especially my supervisors, Dr. R.S. Jones and Mr. D.E. Hall for their guidance, Professor D.V. Parke, former Head of the Department and now University Professor of Biochemistry for the provision of facilities, and my colleagues and friends within and outside the Department for moral support.

My studies have been made possible by a scholarship award from the Federal Government of Nigeria and a study leave granted by the Director of the National Veterinary Research Institute, Vom, Nigeria, for which I am profoundly grateful.

The support of my family is also acknowledged and appreciated, especially my wife, Oyinkan, my son, Adedoyin and my daughter, Yetunde for their constant and abiding faith through it all.
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CHAPTER ONE

INTRODUCTION
1.1 General

The plant, bracken fern (*Pteridium aquilinum* (L) Kuhn) is generally regarded as a single species with two subspecies - *aquilinum*, found in the Northern Hemisphere and Africa and *caudatum* (L) Bonap, found in the Southern Hemisphere. These two subspecies overlap in the West Indies and Malaysia (Tryon, 1941).

Others however believe that the genus actually consists of five or six distinct species (Pichi-Sermolli et al., 1965; Copeland, 1947). It is thus clear that the issue at stake in the taxonomy of bracken is whether each of the varieties should be treated as such or raised to a higher rank. That *Pteridium* is a specific genus is however not in doubt and it has been classified as follows:-

<table>
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<tr>
<th>Division</th>
<th>Pterophyte</th>
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<td>Class</td>
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<td>Family</td>
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<td>Pteridorideae</td>
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<td>Genus</td>
<td>Pteridium</td>
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<td>Species</td>
<td>Aquilinum</td>
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It is an ubiquitous plant ranging from the Arctic circle in Norway to the mid tropics. It has a preference for light acid soils, but may be found in woodlands, thickets, grassy places, open hillsides, peaty soils, burned over areas, is especially adept at taking over "abandoned ground" (Atkinson, 1923; Tryon, 1941) and has been known to grow at areas ranging from sea level to 700 metres in the British Isles and to 3,000 metres on the Continent.

It is a perennial, ranging in height from 1 to 13 feet, has an extensive root stock system (rhizome) which enables it to colonise a wide variety of habitats speedily and ensures that it survives adverse weather conditions like drought and severe environmental influences like fire (Vogl, 1964). It has a stout, erect stem, its leaves (fronds) are broadly triangular with pinnules oblong in the apices of the pinnae.
PLATE I - Bracken (Pteridium aquilinum)

PLATE II - Bracken (Pteridium aquilinum) showing how it colonises the undergrowth
Apart from the creeping rhizome system which enables it to take up water and nutrients readily and in greater quantities thus depriving other competing plants of a means of survival, other factors responsible for its survival include the vast array of chemical constituents assembled in bracken which ensure that it is protected from potential predators (Parrow, 1917; Long and Fenton, 1938). It was shown that rabbits for example tended to consume other plants, thus ensuring the survival of bracken (Watt, 1955). Of all the factors that ensure its survival however the most important would appear to be its ability to release toxic compounds into the environment to retard or "smother" its competitors. This concept recognised as "allelopathy" (Rice, 1974) has being the main factor in making bracken an agricultural problem as well as a veterinary and public health problem.

1.2 Bracken as an Agricultural Problem

Although bracken has long been recognised as part of the native flora in the British Isles, it has become more widespread as the effects of man's activities have become more intensive. As the forest canopy has been removed due to increased demand for wood and space, bracken has been able to thrive better since it has a greater spore output in open areas than in shaded areas (Page, 1982).

Its ability grow well in all types of habitats means that there is less available land for agricultural purposes. In Scotland, for example, it is estimated that about 187,500 hectares of land is bracken-infested and about half of this is well drained prime agricultural land (MacLeod, 1982).

In addition, bracken-intoxicated cattle yield less milk and the milk so produced may be tainted and is thus rejected as unfit for human consumption with loss of income for the farmer (Cooper and Johnson, 1984).

1.3 Bracken as a Veterinary Problem

The drought which affected the British Isles in the spring and summer of 1893 made grazing pastures for livestock sparse and led to the first reported outbreaks of bracken toxicity in livestock (Storrar, 1893; Penberthy, 1893).
The acute clinico-pathologic signs in cattle were described as hyperpyrexia (106°F-108°F), anorexia, depression, cachexia, blood stained faeces, bloody discharges from eyes, nostrils and vulvae, laryngitis, palpitations and increased pulse and respiratory rates, slight jaundice and ulcerations of mucosae and udder.

Although therapy was instituted it was of little avail and it was believed that recovery where it occurred was more fortuitous rather than due to the effects of any medicinal treatment. In most cases death supervened.

At necropsy, the lungs were congested as were the rumen, omasum and abomasum, small and large intestines, with "zebra marking" of the rectum. There were sub-endocardial and sub-pericardial blood staining, cirrhosis of the liver, distension of the gall bladder, hyperaemia of the urinary bladder and congestion of the kidneys. The spleen was normal in size and consistency as well as colour.

Attempts to isolate an infectious agent failed and the only significant guide for the field veterinary officers was the observation that the tips of the young bracken fronds had been bitten off. This led to the speculation that bracken might be responsible for this syndrome and opened an era of intensive investigative activity to delineate the problem of bracken toxicity.

1.3.1 Bovine Studies

The reports of Penberthy (1893) and Storrar (1893) were confirmed by experiments which showed that cows fed bracken developed a haemorrhagic syndrome (Almond, 1894).

The initial studies of Stockman (1909; 1910) were considered unsuccessful because he used small quantities of bracken for rather short periods, but his later studies reproduced the clinico-pathologic signs reported by earlier workers (Stockman, 1917; 1922). He also inferred that the bracken toxin was present in small amounts, required a latent period before it could exert its effects and because of its haemorrhagic effects, he also inferred that it was similar to abrin or ricin. He also suggested that it was heat-resistant because the effects of the toxin persisted in
spite of the pyrexia in affected cattle. A further contribution of Stockman's investigations was to establish that the haemorrhagic syndrome in bracken-intoxicated cattle was not due to avitaminosis C.

The investigations of Shearer (1945) confirmed the clinico-pathologic signs of bracken toxicity in cattle and also suggested that the tannin content of bracken which was found to be a catechol tannin might be responsible for the toxic effects of bracken.

Apart from experimental studies, there were observations made by agriculturists and other scientists which aided the understanding of bracken toxicity in cattle. It was shown that the rhizome could be toxic (Gleeson, 1944; Langley, 1944). Furthermore, two forms of acute bracken toxicity in cattle were described - the enteric form in which there is depression, pyrexia, a weak pulse, enteritis, blood clots in the faeces and pallid conjunctivae, and the laryngitic form characterised by swelling of the throat region, roaring, dyspnoea, increased respiratory rates and pyrexia (Fletcher, 1944).

An important epidemiological observation was made by Boddie (1947) who noted that most of the deaths in Scottish cattle occurred amongst the cattle on the bracken-infested highland regions which were relatively bracken-free. He attributed this to the Hill Cattle Subsidy Scheme of 1946 which required that cattle had to remain on the highlands for 16 weeks continuously for the owners to qualify for subsidy. Prior to the scheme, many owners had brought their cattle to the lowlands for three-weekly intervals and noted that losses had been minimal or non-existent.

Aside from the clinico-pathologic aspects of the disease in cows, biochemical studies revealed that bracken-intoxicated cows had lowered thiamine levels in their sera, an observation which was prompted by similar observations in rats (Evans and Evans, 1949).

Attention was also paid to the haemorrhagic syndrome in bracken toxicity. Naftalin and Cushman (1951) reported that thrombocytopenia and leucopenia were the haematological lesions in bracken-intoxicated cows. However, they found that the packed cell volume and the haemoglobin levels were not affected (Naftalin and Cushman, 1956), but there was regenerative bone marrow damage as shown by the appearance of primitive cells late in the
course of the disease. The persistence of the haematological syndrome in spite of thiamine therapy and supplementation of the diet with vitamin C-containing swedes and hay convinced them that a chemical toxin rather than a nutritional factor was responsible for the toxic signs observed.

Further haematological work by Heath and Wood (1958) showed that the only significant factor responsible for the haemorrhages was thrombocytopenia. They found that thrombin and fibrinogen were abnormally high in bracken-intoxicated cows. These workers also inferred that the leucopenia might be responsible for the profuse bleeding seen in bracken-intoxicated calves. This process might work through the proteolytic action of plasminogen activators produced by amongst other tissues and cell types, leucocytes. These would split peptide linkages in fibrinogen and fibrin, thus promoting the lysis of clots. This process is however not necessarily dependent on the number of leucocytes as on the type and nature of the leucocytes. In this regard they likened bracken toxicity to radiation sickness. In so doing they were to open an entire new area of the toxicology of bracken – the possible carcinogenicity of bracken.

1.3.2 Equine Studies

The toxicity of bracken to horses was reported by Muller (1897), Chesnut and Wilcox (1901) and Pott (1907) but the experimental work of Hawden and Bruce (1917) was the first demonstrated proof that the disease called "Equine staggers" was due to the ingestion of bracken. The clinico-pathologic signs of acute bracken toxicity in the horses were progressive incoordination, hard breathing, papillary dilation, loose faeces and rapid pulse. At necropsy the brain was apparently congested as were the kidney cortices, but there were no other significant gross pathological lesions.

Further studies reproduced the clinico-pathologic signs and in addition blood thiamine was observed to drop steadily, pyruvate to rise steadily, leucocytosis to occur early in the course of the disease but to return to normal later, eosinopenia occurred during the staggering episodes and thrombocytopenia occurred but were reversed by thiamine therapy (Evans et al., 1951).
At necropsy, there were tympanitis, congestion of the liver, petechiations of the spleen, excessive pericardial effusions, petechiations of the myocardium, a flabby right ventricle, congested kidneys, and bruising and oedema of the sciatic nerve.

Histopathology revealed degeneration of the Purkinje fibres and disappearance of their nuclei.

The key symptom of bracken toxicity in the horse is neurological and the equine staggers can be corrected by thiamine therapy giving rise to the hypothesis that bracken contains a thiaminase or "antithiamine factor".

1.3.3 Ovine Studies

The report of a suspected case of bracken toxicity in sheep in New Zealand (Hickman, 1910) was regarded with scepticism. Other reports however followed, indicating that though sheep were susceptible to bracken, they were more resistant than cattle (Sampson and Malmsten, 1935; Perkins, 1950; Foggie, 1951).

It was however, the experimental studies of Moon and Raafat (1951a) which showed conclusively that sheep developed signs upon ingesting bracken similar to those of cattle intoxicated by bracken. Sheep developed pyrexia (104°C), foetid, blood-stained faeces and at necropsy there were petechiations of the gut, kidneys, spleen and heart. The lungs had a purple mottled appearance.

In the bracken-infested North York moors, sheep involved in an outbreak had leucopenia and thrombocytopenia, blood-stained faeces, hyperpyrexia (102.4°F - 106°F) and at autopsy there were haemorrhages in the stomach, small and large intestines, on the myocardium, spleen, kidneys, subcutis and serosa (Parker and McCrea, 1965).

Another sign seen in bracken-intoxicated sheep was progressive retinopathy or "bright blindness" (Watson et al., 1965). This sign has also been confirmed by a subsequent report, which also indicated that the lactate dehydrogenase levels in the retinae of bracken-intoxicated sheep were lower than in unaffected sheep (Watson et al., 1972).
When the bracken rhizome which is more toxic than the fronds was fed to sheep they developed ataxia in addition to leucopenia, thrombocytopenia and lowered blood pyruvate. When treated with thiamine, they responded dramatically but if left untreated, they developed cerebro-cortical necrosis. It was concluded that the antithiamine factor which was found in greater quantities in the rhizome than the fronds was responsible for this lesion (Evans et al., 1975).

The clinical pathologic picture of bracken toxicity in sheep would therefore include progressive retinopathy, bone marrow aplasia resulting in a haematological syndrome, and cerebro-cortical necrosis probably due to avitaminosis B1.

It would however appear that sheep are less susceptible to bracken toxicity than cattle. The reason for this is uncertain, but it may be due to a more discerning eating habit in sheep than cattle which enables them to avoid bracken more easily.

1.3.4 Porcine Studies

Although Forsyth (1954) reported that bracken toxicity in pigs was due to eating fronds and not the rhizomes, that report has never been corroborated. Attempts to induce bracken toxicity in pigs with bracken fronds have been ineffective while pigs fed the rhizome developed avitaminosis B1, elevated blood pyruvate, anorexia and listlessness but not leucopenia nor thrombocytopenia. There was however a drop in reticulocytes which was corrected by the institution of thiamine therapy, and it was inferred that thiamine played a central role in red blood cell formation in monogastrics. It is noteworthy that though the thiamine levels dropped, there was no ataxia or nervous degeneration. At necropsy the heart was mottled and dilated, the lungs oedematous, the trachea frothy and the gall bladder enlarged (Evans et al., 1963; Evans et al., 1972; Harding, 1972).

It would thus appear that though pigs are highly susceptible to the antithiamine factor in bracken they do not develop haematological changes. This raises the possibility of two distinct poisons in bracken - the antithiamine factor which in monogastrics would induce ataxia and the bone
marrow toxin which in ruminants and monogastrics would induce bone marrow depression. Further studies were required to clarify these issues and these were to be done in laboratory animals.

1.4 Laboratory Animal Studies

The advantages of using laboratory animals for toxicological studies lie in the cost, reproducibility and speed with which studies can often be carried out.

The clinico-pathologic signs of feeding bracken to rats were anorexia, ataxia and bone marrow damage resulting in a haemorrhagic syndrome. Biochemical analyses revealed low tissue thiamine levels, high blood pyruvate and a dysfunction of thiamine pyrophosphate dependent enzymes. There were thrombocytopenia and leucopenia when black rats were used and at necropsy, there was atrophy of the hair follicles, epidermis and sebaceous glands. These signs could not be reproduced when autoclaved bracken was used suggesting that the bracken toxin was susceptible to heat (Weswig et al., 1946; Evans and Evans, 1949; Naftalin and Cushnie, 1951; Cordy, 1952). It was shown by Evans and Evans (1949) that 0.1 mg thiamine administered subcutaneously alleviated the clinical and pathological signs of bracken toxicity in the rat.

1.5 The Treatment of Bracken Toxicity

From the earliest reported cases of bracken toxicity, it was imperative that a means of clinical management had to be devised in order to minimise losses in livestock. However, the lack of certainty as to the nature of the poison proved an obstacle and made attempts at therapy largely unsuccessful (Storrar, 1893; Fletcher, 1944). The view was taken by Stockman (1917) that preventative rather than therapeutic measures would be a better way of controlling bracken toxicity.

Thiamine therapy was however useful especially with regard to cases in horses, rats, pigs and if administered early, to sheep (Evans et al., 1951; Ueda et al., 1950; Carpenter et al., 1950; Evans and Evans, 1949; Cordy, 1952; Evans, 1976; Harding, 1972). However, once "bright blindness" had
developed in sheep along with cerebro-cortical necrosis, thiamine therapy was useless. Combined antibiotic - DL batyl alcohol therapy was also attempted with some success (Evans et al., 1958).

When it was observed that bracken toxicity led to the release of histamines and other amines and factors indicative of a strong immunological involvement, corticosteroid therapy was attempted with some success (Evans, 1968).

However, the best approach to the bracken problem is preventative and eradication of bracken would be a useful adjunct to this as well as denying livestock access to bracken infested areas.

In attempting to achieve eradication of bracken, the herbicide, asulam (methyl 4-aminobenzene-sulphonyl carbamate) has been the most effective. Ploughing is usually difficult because of the terrain and the extensive rhizome is further certain to frustrate efforts in this direction. Burning is equally ineffective due in part to spore-formation (Holroyd, 1971), and the extensive underground rhizome.

1.6 The Carcinogenicity of Bracken

The acute toxicity of bracken is characterised by bone marrow aplasia resulting in leucopenia and thrombocytopenia. In addition, primary damage occurs in the intestinal mucosa (Evans et al., 1951, 1954; Naftalin and Cushnie, 1951, 1954).

These observations led workers to suggest that the bracken toxin was an antimitotic agent acting at the sites of the most rapid cell division - the gut and bone marrow. It is possible to envisage a mechanism of action for the toxin which simultaneously affects the bone marrow and the intestinal tract which is then manifested as a haemorrhagic syndrome. The depression of the bone marrow would lead to a reduction in the vascular system of the formed elements of blood while the destruction of the gut mucosa which contains large amounts of serotonin would lead to lack of capillary stability with resulting extravasation of blood.
This mode of action is identical to category 3 of the radiation syndrome (Table 1.1) (Evans et al., 1958; Heath and Wood, 1958; Schultze et al., 1959; Brown et al., 1961; Brown, 1962; Evans, 1968).

It would be expected that if bracken toxicity was like the radiation syndrome, appropriate doses of the bracken toxin administered over an appropriate period would eventually result in category 4 of the radiation syndrome - that is eventually result in cancer (Table 1.1).

That the bracken toxin was carcinogenic was confirmed by a report which showed that after feeding five cows on green as well as dry bracken, four developed haematuria and "changes of a polypous-tumorous nature" in their urinary bladders (Rosenberger and Heeschen, 1960).

This report led various workers to begin investigative work on the possible carcinogenicity of bracken.

1.6.1 Bovine Studies

The finding of haematuria and bladder tumours in cows from the bracken-infested Bolu District of Turkey was thought but not conclusively proved to be due to bracken (Pamukcu, 1963). This condition, called enzootic bovine haematuria (EBH) was thought to be due to a variety of aetiological agents of which bracken was to prove to be the most likely.

Other studies soon reproduced the symptoms of EBH, haemangiomas, papillomas and haematuria in the bladders of cows fed bracken (Sofrenovic et al., 1965; Rosenberger, 1965; Pamukcu et al., 1967; Price and Pamukcu, 1968).

These observations would suggest that bracken is carcinogenic, with the bladder and the small intestine being the target organs.

They also suggest a role for bracken in EBH but as will be seen, it was probably not the only causative factor of EBH.
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<td>Weeks</td>
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<td>Gastrointestinal</td>
<td>Bone marrow death</td>
<td>Cancer death</td>
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<td>system death</td>
<td>tract death</td>
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<td>b) whole body</td>
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<td>Signs</td>
<td>Immediate death</td>
<td>Electrolyte loss</td>
<td>Delayed gastrointestinal syndrome</td>
<td>Induction of mutation</td>
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<td>Systemic toxaemia</td>
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<td>Histamine and heparin release</td>
<td>Capillary fragility</td>
<td>Sterility</td>
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<tr>
<td></td>
<td>Capillary fragility</td>
<td>Humoral changes including lipids.</td>
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<td>Decreasing dosage of radiation</td>
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(After Evans, 1968)
Enzootic bovine haematuria (EBH) is a chronic, non-infectious disease of cattle, characterised by the development of haemangiomatous lesions of the wall of the urinary bladder and clinically by haematuria resulting in anaemia and death (Blood et al., 1979). It is ubiquitous in occurrence but in each case, it is restricted to specific areas and is enzootic in nature (Figure 1.1).

The aetiology of EBH has been the subject of much investigation but even now remains undecided. The initial aetiological factor was thought to be nutritional but this was not supported by experimental evidence unlike the other aetiological factors such as non-specific irritations (mechanical, mycotic or toxic), toxic plants (oak, ash, privet, hornbean, hazel, dog-berry, pine and fern), trauma due to filaria, distoma or pentastomes, coccidia and bacteria.

The possible bacterial aetiology was examined but the failure to isolate any bacteria from the urine and internal organs of the early cases, and the failure to reproduce the disease by injecting the urine and blood of affected cases into experimental cases eventually led to the bacterial aetiology being considered implausible (Hawden, 1917).

The finding of adult *Schistosoma japonicum* in the bladder of two cases in Formosa suggested that *Schistosoma* spp. may have a role in the causation of EBH but five other cases had no *Schistosoma* in their bladders (Ichikawa, 1922).

Oxalic acid has been suggested as a cause of EBH. It was reproduced both by oral drenching and by injection of oxalic acid into the urinary bladder, the oxalic acid being in the form of calcium oxalate (Hawden, 1917). This work is supported by the knowledge that the clinical signs of oxalate poisoning in cattle include haematuria and bloody nasal discharges (Blood et al., 1979).

The feeding of the oxalate-containing plants *Psicalon absimile* to rabbits failed to reproduce the disease (Rimington and Steyn, 1933) as did not the feeding of *Oxalis cernua* to sheep (Bull, 1929) nor the feeding of *Rumex acetosa* to cattle (Craig and Kehoe, 1921).
The oxalate theory of Hawden (1917) was not corroborated by experimental evidence. It has been suggested that in the area of Canada where Hawden's observations were made, there are not many oxalate containing plants. It may be that he was able to produce signs identical to EBH because of very high doses of oxalic acid which he used, which would not occur in a natural situation. The oxalate theory of EBH causation is also implausible.

Observations from Australia that phosphorus and manganese deficiency occurred in areas of high EBH incidence tended to support a chemical deficiency theory of the causation of EBH (Bull et al., 1932). Earlier however, blood analysis failed to show any chemical or dietary deficiencies (Fleming et al., 1930). It is plausible to suggest that dietary deficiencies resulting in certain chemical deficiencies would aid the course of any disease but it is unlikely that EBH is caused by manganese and phosphorus deficiency acting on their own.

A protozoan parasite belonging to the genus *Entamoeba* isolated from the urine of EBH cases was suggested as the cause of the disease (Datta, 1934). In later reports however it was reclassified as *Aspergillus* and was thought to exist in a symbiotic relationship with lichens and bracken (Datta, 1952; 1953). Thus it appears that the organism is merely an incidental finding and that bracken is the real cause of EBH.

The observation in Turkey that haematuria occurred mainly in the bracken-infested districts would suggest that it was the main cause of EBH (Pamukcu, 1955). Further reports seemed to confirm bracken as the main and most consistent factor in all cases of EBH either observed in field conditions and justified epidemiologically or reproduced experimentally (Rosenberger and Heeschen, 1960; Pamukcu, 1963; Sofrenovic et al., 1965).

The possibility that other factors may co-operate with bracken to produce the disease has not been ruled out especially in the light of the report of Jarrett et al. (1978) which suggests that a papilloma virus might also be implicated. This virus had earlier been considered a passenger virus not central to the causation of the disease (Olsen et al., 1965) but recently a bovine papilloma virus, BPV-2, has been isolated from urinary bladder neoplasms associated with EBH (Lancaster and Olsen, 1982; Jarrett, W.F.H., cited by Hopkins, 1986). Although BPV-2 is associated with benign
lesions, it is thought that the fibropapilloma so caused may progress to malignancy to form carcinomas due to the effects of a variety of environmental and genetic stimuli of which bracken may be one (Smith and Campo, 1985).

Thus the main aetiological agent of EBH is bracken but other factors might contribute to the course and prognosis of the disease, that is, there is a multifactorial causation of EBH.

1.6.2 Ovine Studies

Although bracken had been linked to sheep tumours (Dodd, 1960; McDonald and Leaver, 1965; McCrea and Head, 1978) it was the experimental work of McCrea and Head (1981) that demonstrated this conclusively. In the field cases which they reported (McCrea and Head, 1978) they found fibrosarcomas, fibropapillomas, a squamous cell papilloma, squamous cell carcinomas, adenocarcinomas, primary carcinomas, a lymphosarcoma and a thymoma, compared to their experimental cases where transitional cell carcinomas, fibrosarcomas and one rumen papilloma were found. Furthermore the field cases did not show any bladder involvement while the experimental cases did and they concluded that other factors such as the liver fluke might have added to the wide variety of tumour types seen in the field cases. The ability of the liver fluke, Fasciola, to act as a tumour promoter has been demonstrated by Flavell and Lucas (1982, 1983). The louping-ill virus, and the papilloma virus were also considered to be contributory factors to the wider variety of tumours seen in field cases compared to those in the controlled experimental situation.

1.7 Laboratory Animal Studies

The reports of carcinogenicity due to bracken in livestock could only be confirmed by using large numbers of animals. Laboratory animals were ideal for this purpose.
1.7.1 Studies in Rats

The carcinogenicity of bracken has been demonstrated in rats by various studies which showed that ileal adenocarcinomas, adenomas and sarcomas, bladder papillomas, sessile or papillary carcinomas, transitional cell carcinomas, adenocarcinomas, fibromas, leiomyosarcomas and haemangiomas, mammary adenocarcinomas and fibroadenomas as well as hyperplastic liver foci could be induced in rats by feeding bracken-containing diets (Mason, 1965; Evans and Mason, 1965; Price and Pamukcu, 1968; Pamukcu and Price, 1969; Schacham et al., 1970; Pamukcu et al., 1970, 1971, 1976; Hirono et al., 1970, 1972, 1973, 1983, 1984a; Yunoki et al., 1973; Kawai et al., 1981; Yoshikawa et al., 1981; Erturk et al., 1983).

The ileum was the most common site of bracken induced tumours in rats followed by the urinary bladder. The involvement of the mammary glands and liver have been shown in isolated reports.

1.7.2 Studies in Mice

The bracken-induced tumours of mice include pulmonary adenomas and adenocarcinomas, urinary bladder carcinomas, intestinal carcinomas, gastric carcinomas, hepatomas and leukaemias (Evans and Widdop, 1966; Evans, 1968; Evans et al., 1968, 1969; Pamukcu et al., 1972, 1977; Jones, 1974; Miyakawa and Yoshida, 1975; Hirono et al., 1975).

1.7.3 Studies in the Quail (Coturnix coturnix japonica)

Adenocarcinomas of the caecae, colon and ileum were observed in Japanese quail fed an active bracken extract (Evans et al., 1967; Evans, 1968).

1.7.4 Studies in the Hamster

Caecal and ileal adenocarcinomas were found in four of twenty-four Syrian Golden Hamsters fed a 30% by weight bracken diet (Evans, 1968; Kawai et al., 1979).
1.7.5 Studies in the Guinea-Pig

Bracken-intoxicated guinea-pigs developed haematuria, accompanied by carcinomas and infiltrating adenocarcinomas of the urinary bladder. In some cases intestinal adenocarcinomas developed (Evans et al., 1967; Evans, 1968) (Appendix I; Appendix II).

It is pertinent at this stage to summarise the species differences with regard to the toxicity of bracken. The simple-stomached animals like the horse, pig and rat develop a neurological ataxia after 28 days on a bracken diet. This ataxia syndrome has been attributed to the destruction of thiamine in the monogastrics by the enzyme thiaminase found in bracken.

In the ruminants, however, an exogenous source of thiamine is not essential as they are able to synthesise thiamine from their rumen microflora. Although they have succumbed to an avitaminosis B₁ syndrome due to feeding of bracken rhizomes, the symptoms were not those of a neurological ataxia but cerebrocortical necrosis.

Both the neurological ataxia of monogastrics and the cerebrocortical necrosis of ruminants respond to thiamine therapy.

Furthermore, there is a haematological syndrome which manifests as part of the acute toxicity of bracken in the ruminants (though less severely in sheep). The monogastrics are however less susceptible to the bone-marrow toxin which affects the ruminants more readily. It is thought that the bone marrow toxin is different from thiaminase.

The chronic toxicity of bracken in both monogastrics and ruminants is the production of a variety of tumours. So far, however, no tumours have been reported in pigs and horses.

It is also not certain if it is the same toxin that produces the bone marrow damage and the tumours.
1.8 Factors influencing the Carcinogenicity of Bracken

1.8.1 Phytomerial and Ecological Factors

Comparative studies showed that the curled tops of young bracken had more potent carcinogenic activity than the stalks, and the carcinogenicity of the rhizomes was greater than that of the fronds. In addition, the young frond was more carcinogenic than the mature plant. The rats on a diet of mature bracken had a longer latent period before induction of cancer than those on a diet of young bracken. Furthermore, the young fronds tended to induce adenocarcinomas while the mature fronds tended to induce adenomas in the ileum, but rats fed the mature bracken tended to have more urinary bladder tumours than those fed the young frond (Hirono et al., 1973; Pamukcu et al., 1977).

The geographical location of the bracken also influences its carcinogenicity (Hirono et al., 1972).

1.8.2 The Effects of Animal Models on Carcinogenicity

The age, species and strain of laboratory animals used for bracken carcinogenicity tests have an effect on the nature of the tumours produced.

Young animals appear to be more susceptible than older animals (Evans, 1968), F344 rats had a higher tendency to produce bladder tumours (96%) when fed bracken than did Sprague Dawley rats (46%) (Erturk et al., 1983), C57Bl/6 mice tended to develop jejunal tumours when fed bracken but Swiss mice tended to develop pulmonary adenomas and it would appear that mice are more susceptible to the haematological disorders induced by bracken than are rats. Whereas leukaemias have been found in mice they have not been reported in rats (Pamukcu et al., 1972).

Neither the sex of the animal nor presence, absence or nature of the gut flora are thought to be of significance in bracken-induced carcinogenicity (Sumi et al., 1981). This is in contrast to the bracken constituents, quercetin and shikimate whose metabolism is strongly dependent on the presence or absence and nature of the gut flora (Nakagawa et al., 1965; Brewster et al., 1978).
1.8.3 Chemicals and Drugs affecting Bracken Carcinogenicity

When bracken-fed rats were given thiamine at 2 mg per rat per week, the incidence of urinary bladder tumours was higher than for rats fed bracken only (Pamukcu et al., 1970b). The explanation put forward by the authors that thiamine altered the absorption, distribution and metabolism of the bracken carcinogen may be plausible if the role of thiamine with respect to the metabolism of some bladder carcinogens and bladder tumour promoters is considered. Some tryptophan metabolites like xanthurenic acid and kynurenic acid were demonstrated to produce bladder carcinoma in mice (Allen et al., 1957). These metabolites are part of the kynurenine pathway of tryptophan metabolism. The first step in this reaction in which tryptophan is converted to N-formyl kynurenine requires thiamine as a co-factor for the enzyme tryptophan pyrrolase (Dalgleish, 1955). In the absence of thiamine these metabolites are not produced (Dalgleish, 1955). Thiamine supplementation may therefore promote the production of these bladder carcinogens.

Phenothiazine reduces the incidence of both urinary bladder and intestinal tumours in bracken-fed rats (Pamukcu et al., 1971). They suggested that this action might be due to the induction of the mixed function oxidase system by phenothiazine resulting in the detoxification of the bracken carcinogen. They also suggested a direct inhibitory effect of phenothiazine on the carcinogen. Another explanation could be the cathartic action of phenothiazine which reduces the residence time of the bracken carcinogen in the gut. This explanation is supported by the work of Hirono et al. (1977b) which showed that bracken-induced tumours tended to appear in the parts of the gut most prone to intestinal stasis.

The antioxidants, disulfiram and butylated hydroxyanisole have been shown to inhibit bracken-induced carcinogenic activity in the intestinal tract probably via a mechanism that prevents autoxidation, while polyvinylpyrrolidone and calcium chloride were shown to reduce the incidence of bracken-induced bladder cancer possibly by forming insoluble complexes with the tannins and flavonoids in bracken (Pamukcu et al., 1977).

Nitrite and nitrate supplementation increased the incidence of urinary bladder tumours in bracken-fed rats (Erturk et al., 1974). It may be that this was mediated via the formation of N-nitroso compounds.
Finally, if bracken is boiled with wood ash, sodium bicarbonate and sodium chloride, its carcinogenic potency is reduced (Hirono et al., 1972). The increased alkalinity of the intestinal tract due to the feeding of bicarbonate was suggested as the factor responsible for the reduced intestinal tumours. However, the incidence of bladder tumours in bicarbonate fed rats increased (Erturk et al., 1974; Pamucku et al., 1977).

These studies on the effects of various chemicals on the incidence of tumours in bracken intoxicated animals lead to some conclusions about the nature and mechanism of action of the bracken carcinogen. The effect of phenothiazine would be to suggest that the bracken carcinogen may be metabolised via a pathway dependent on cytochrome p450 but in the same vein, the possibility of a purely physical effect in which contact time between carcinogen and organ is reduced has to be considered. A possibility which is reinforced by the effect of calcium chloride and PVP.

The effects of the antioxidants BHA and disulfiram are suggestive of a role for the process of autoxidation as one of the possible mediating mechanisms for bracken carcinogenesis, raising the possibility of the involvement of free radicals. This possibility is reinforced by the work of Evans (1968) which reveals the presence of immunologically active cells in the tissues including those with phagocytic activity believed to involve the release of free radicals. Antioxidants such as BHA act to interrupt the free radical chain of oxidative reactions by contributing hydrogen from the phenolic hydroxyl groups, and in the process forming stable free radicals which do not initiate nor propagate further oxidation of lipids.

The role of nitrite and nitrate and the suggested implication of N-nitrosation as another possible mechanism for the mediation of bracken carcinogenicity is an important point in bracken carcinogenesis.

By far the most important point illustrated by these apparently disparate possibilities is the complex nature of bracken-induced carcinogenesis and the possibility that there is a multifactorial causation of disease in bracken-induced toxicity. The various aetiological factors or mechanisms may entail a role for free radicals and autoxidation, for N-nitrosation and N-nitroso compounds, and a role for the anti-thiamine factor and an alteration of the metabolism of tryptophan.
It also illustrates the difficulty entailed in making firm comments on the mechanism of bracken carcinogenesis without knowing the identity of the bracken carcinogen.

1.9 The Teratogenicity of Bracken

When pregnant ICR-JCL mice were fed a 33% bracken diet, there was weight loss, intra-uterine growth suppression and retarded ossification of the sternebrae (Yasuda et al., 1974). In C57BL/6 mice there was an increased frequency of abortions (Fushimi et al., 1973).

1.10 Bracken as a Public Health Problem

In some parts of Europe, the bracken rhizome was ground and baked into bread for human consumption (Medsger, 1939), while in North America, the Indians of the Pacific coast used it for food after boiling in water. In New Zealand, the Maoris used it for food after treating it similarly. In Japan, where the stalks and fronds are processed for human consumption by immersion in boiling water containing wood ash or sodium bicarbonate or by pickling in salt and immersion in boiling water (Anon., 1943; Fernald and Kinsey, 1943; Anon., 1967; Shawcross, 1967; Evans, 1976; Hirono et al., 1972; Sugimura, 1976; Pamukcu et al., 1977), it was one of the factors identified as responsible for increasing the relative risk to oesophageal cancer (Hirayama, 1979). In Norway and Siberia, the stalks and fronds were fermented into beer (Harrington, 1967).

The other source of public health hazard is the consumption of milk and milk products from cows grazing in bracken-infested fields. The milk of bracken-fed cows was mutagenic to Salmonella typhimurium TA100, and also induced intestinal, renal and urinary bladder carcinomas in rats (Pamukcu et al., 1978).

The possibility of rain leaching bracken carcinogens into the underground water supply from the bracken-infested areas has also been suggested (Boyland, 1967; Evans et al., 1971).
Another possibility recently raised is the carcinogenicity of the bracken spores, which upon inhalation may pass through the alveoli into the stomach and possibly cause stomach cancer (Evans, I.A. in press).

The geographical correlation between human oesophageal and gastric cancers, EBH and acute bracken poisoning is illustrated in Figure 1.1.

1.11 The Chemical Composition of Bracken

Interest in the chemical composition of bracken arose from its observed toxicity to livestock and the potential public health hazard which it may pose.

Some of the chemical constituents of bracken have no known direct toxic effect on livestock but may aid the plant to survive in a wide variety of environments.

The constituents of the plant known to be of some biological significance include an antheridogen (Pringle et al., 1960) and the insect hormones ecdysone and 20-hydroxyecdysone (Kaplanis et al., 1967). The presence of a thiaminase has also been reported. This enzyme, the first of its kind to be reported in the plant kingdom, is concentrated in the rhizome although the fronds contain some also. It is considered responsible for the neurological signs seen in bracken-fed monogastrics and the cerebro-cortical necrosis of sheep (Evans and Evans, 1949; Weswig et al., 1946; Hawden and Bruce, 1933; Evans et al., 1950, 1952; Evans et al., 1951; Evans et al., 1972, 1975; Watkin et al., 1953; Thomas et al., 1957; Harding, 1972; Kelleway and Geovjian, 1978; Ueda et al., 1950).

Although the cyanogenic glycoside, prunasin is a constituent of bracken (Kofod and Eyjolfsson, 1966; Cooper-Driver and Swain, 1976) it has not been ascribed any toxic effects in livestock (Moon and Raafat, 1951b). However, it has been suggested that it plays a role in predator prevention (Cooper-Driver and Swain, 1976).
Figure 1.1

AREAS of HIGH INCIDENCE

△ ACUTE BRACKEN POISONING
● ENZOOTIC BOVINE HAEMATURIA

□ STOMACH CANCER
▼ OESOPHAGEAL CANCER

CATTLE

MAN

(After Jones, 1974)
1.12 The Carcinogenic Constituent(s) of Bracken

Greater attention has been paid to the nature and identity of the bracken carcinogen. To this end various groups of compounds have been extracted from bracken and tested for carcinogenic activity in a variety of animal models and cell systems. Bracken isolates that have been considered for their carcinogenic potential include flavonoids, terpenoids, tannins and phenolic acids (Appendix III).

1.12.1 The Flavonoid Constituents of Bracken

Flavonoids are secondary plant constituents, very widely distributed in ferns. The flavonol glycosides, kaempferol-3-glycoside (astragalin), quercetin-3-rhamnoglucoside (rutin), quercetin-3-glucoside (isoquercitrin), kaempferol-3-p-coumaryl glucoside (tiliroside), 3,3',4',5,7-pentahydroxy-flavone (quercetin) and its glycosidic derivative, quercitrin, have all been isolated from bracken (Nakabayashi, 1955; Wang et al., 1973; Pamukcu and Bryan, 1979; Pamukcu et al., 1980b).

Tiliroside was tested for carcinogenic activity in Swiss albino mice by intravesical instillation but the results were negative (Wang et al., 1976) as were mutagenicity tests with Salmonella typhimurium TA 98 and TA 100, with or without the S-9 activating system. However, after pre-incubation with hesperidinase, the released aglycone, was mutagenic to both S. typhimurium TA 98 and TA 100 in the presence of the S-9 mix (Nagao et al., 1981).

Rutin was not mutagenic in the Ames' test using S. typhimurium TA 98, TA 100, TA 1535, TA 1537 and TA 1538 (Brown and Dietrich, 1979). Upon hydrolysis by glycosidase however, it is activated to a mutagenic compound, quercetin (MacDonald et al., 1983; Nagao et al., 1981). It was negative in the micronucleus test using male Swiss mice (Sahu et al., 1981) and was not carcinogenic when tested as a 10% diet in male and female non-inbred golden hamsters (Morino et al., 1982).

Isoquercitrin was non-mutagenic to S. typhimurium TA 98 and TA 100 except when pre-incubated with hesperidinase (Nagao et al., 1981).
Astragalin was also non-mutagenic to *S. typhimurium* TA 98 and TA 100 both with and without the S-9 mix, but on incubation with hesperidinase it was mutagenic to *S. typhimurium* TA 98 and TA 100 with S-9 activation.

Although kaempferol was mutagenic to *S. typhimurium* TA 98 and TA 100 with S-9 activation (Sugimura et al., 1977; Hardigree and Epler, 1978; MacGregor and Jurd, 1978; Nagao et al., 1981) to *S. typhimurium* TA 1537 (Brown and Dietrich, 1979), to V79 Chinese hamster cells (Maruta et al., 1979) and was positively clastogenic in the micronucleus test (Sahu et al., 1981) it was not carcinogenic when fed as a 0.04% diet to Fischer 344 (F344) rats for 540 days (Takanashi et al., 1983).

Quercetin is mutagenic to *Salmonella typhimurium* TA 98 (Hardigree and Epler, 1978; Seino et al., 1978) to TA 98 and TA 100 (Bjeldanes and Chang, 1977; Sugimura et al., 1977; MacGregor and Jurd, 1978; Nagao et al., 1981) to TA 98, TA 100, TA 1537 and TA 1538 (Brown and Dietrich, 1979) without activation with the S-9. It was clastogenic to male mice (Sahu et al., 1981), weakly mutagenic to *Escherichia coli*, *Saccharomyces cerevisiae* (Hardigree and Epler, 1978) and *Drosophila melanogaster* (Watson, 1982), but non-mutagenic to *Bacillus subtilis* (MacGregor and Sachs, 1979). It was also mutagenic to V79 Chinese hamster cells (Maruta et al., 1979) and genotoxic to Chinese hamster ovary (CHO) cells (MacGregor et al., 1980; Carver et al., 1983). It induced single-strand breaks in the DNA of mouse lymphoma L5178Y cells (Meltz and MacGregor, 1981) as well as a dose-dependent increase in sister chromatid exchanges (SCE) in cultured human lymphoblastoid NL3 cells (Sugimura, 1979), HE 2144 human fibroblasts, Don-6 and B-131 Chinese hamster fibroblasts and human lymphocytes (Yoshida et al., 1980).

It is non-teratogenic (Willhite, 1982) but its carcinogenicity is a more equivocal issue. Although some authors claim that it is a urinary bladder, intestinal and liver carcinogen (Pamukcu et al., 1980b; Erturk et al., 1983), others report no such activity (Ambrose et al., 1952; Morino et al., 1982; Takanashi et al., 1983). The varying results from different workers led others to suggest that it might act as an initiator or promoter of carcinogenesis (Kato et al., 1983; Fukushima et al., 1983; Hirose et al., 1983).
Quercetin has a widespread occurrence in the plant kingdom some of which are used for human consumption. It is used as an antioxidant in the food industry and for the improvement of butter quality (Ambrose et al., 1952; Harborne, 1977; Seino et al., 1978; Stitch et al., 1981; Siess and Vernevaut, 1982; Sahu et al., 1981).

1.12.2 The Terpenoid Constituents of Bracken

Sesquiterpenes having the 1-indanone nucleus and called pterosins and their O-glucosides (pterosides) have been isolated from bracken (Hikino et al., 1970; 1971; 1972; Fukuoka et al., 1972; Yoshihira et al., 1972; Kuroyanagi et al., 1974).

They are cytotoxic to HeLa cells but not carcinogetic to rats (Saito et al., 1975).

A lactam isolated by Takatori et al. (1972) called pterolactam was non-carcinogenic (Hirono et al., 1975).

Recently a compound designated aquilide A was isolated from bracken. It is mutagenic in the Ames Salmonella Assay and genotoxic to mammalian cells in vitro. It is unstable being converted into pterosin B under acid conditions and under alkaline conditions into a compound as yet unidentified but closely resembling the pterosins (van der Hoeven et al., 1983). Aquilide is the same as a compound isolated from a carcinogetic fraction of bracken and designated ptaquiloside (Hirono et al., 1984c).

Ptaquiloside was carcinogetic in Sprague-Dawley rats, inducing mammary and intestinal tumours as well as acute bracken poisoning in a calf (Hirono et al., 1984b).

The results from ptaquiloside are interesting and suggest that it might be the bracken carcinogetic, and that the bone marrow toxin and the carcinogetic are the same compound. Certain features of the chemistry and toxicology of ptaquiloside would appear however to render it improbable as the bracken carcinogetic. It is unstable under both acidic and basic conditions, being rapidly converted into pterosin B. This is in contrast to the chemical activity of carcinogetic fractions of bracken (Pamucku et al., 1970a; Evans,
Furthermore, although very high doses of ptaquiloside were used only mild leucopenia was observed in the experimental calf and even this remained within normal limits. The erythrocytes were unaffected, the thrombocytes fell only slightly and haemorrhages were not observed. The sternal marrow remained almost normal and it can be inferred that they did not reproduce the acute disease in the calf.

These do not invalidate the possibility that ptaquiloside may have a role in bracken-induced carcinogenesis, probably an initiating role in view of its mutagenicity in the Ames assay and its genotoxicity to mammalian cells.

1.12.3 The Tannin Content of Bracken

The initial allusions to the involvement of tannins in bracken toxicity were made by Long (1924) and Holmboe (1943) who suggested that "pteritannic acid" was the toxic constituent. Shearer (1945) also suggested that a tannin might be the toxic constituent of bracken. Wang et al. (1976) isolated a tannin from bracken and using intra-vesical implantation were able to demonstrate its carcinogenicity, but this technique is a controversial one. When tested by oral administration however, they failed to induce any tumours. In the same experiment the tannin-free fraction of bracken induced intestinal tumours in rats. When administered subcutaneously however, fibrous histiocytomas were induced by the tannin isolated from bracken (Pamukcu et al., 1980a). Whether the bracken tannin is carcinogenic or just exhibiting a local irritant effect on both the skin and in the bladder is not certain.

Furthermore, the nature of the bracken tannin is uncertain. The term "tannin" refers to a generic term describing heterogenous polymeric substances which differ from plant to plant (Kirby, 1960). Studies with tannin-containing fractions of plants notably Krameria ixina and Acacia villosa demonstrated that tannin was carcinogenic via the dermal route but not via the oral route (O'Gara et al., 1971, 1974).

Epidemiological evidence suggests that the oesophageal cancers of the people of Curacao is due to the high tannin content of their diets. The betel nut Areca catechu, the cause of oral cancers also has a high tannin
content. The Luo people of Kenya who use a high tannin-containing sorghum for their beer have a 14-fold increase in oesophageal cancer compared to the rest of the population. In West Africa where a low tannin sorghum is used, there is a low incidence of oesophageal cancer (Morton, 1978).

The epidemiological evidence notwithstanding the inability to demonstrate the carcinogenicity of tannins via the oral route must cast doubt on the ability of the bracken tannin to induce cancer in bracken-fed animals.

1.12.4 Shikimic Acid

Shikimic acid is a trihydroxycyclohexene carboxylic acid which has been implicated in bracken carcinogenicity (Evans and Osman, 1974). Shikimic acid was first described as a natural product from the plant Illicium religiosum, Sieb. and it was from the Japanese name of this plant, "shikimino-ki", that the name shikimic acid was derived. It is a strategic metabolite in the formation of several aromatic compounds (Haslam, 1974).

Although it was reported to be carcinogenic to mice (Evans and Osman, 1974) it has not been shown to be carcinogenic to rats (Hirono et al., 1977a). In this study however, 3 of the 12 rats on the shikimate diet died of pneumonia at various intervals during the study, while the controls remained healthy throughout the 480 days of the experiment. It would be most appropriate to feed the test compound to the test animals over 2 years before firm conclusions can be reached as regards its carcinogenicity (DHSS, 1982).

It was nonmutagenic in the Ames' test (Jacobsen et al., 1978) but genotoxic in the BHK 21 cell transformation test (Jones et al., 1983). Its metabolites in the rat were neither mutagenic nor genotoxic (Brewster, 1978; Jones et al., 1983).

Shikimate is widespread in nature occurring in several human foods especially fruits, hence the immense interest in it from a public health viewpoint, to determine whether it is a carcinogen or not and to define its mode of biological activity.
1.13 The Nature of the Bracken Carcinogen

The identity of the bracken carcinogen is still uncertain but some features of its nature are known:-
(i) it is soluble in water (Hirono et al., 1978)
(ii) it is insoluble in heptane and ether (Evans, 1979)
(iii) it is soluble in methanol (Pamukcu et al., 1970a)
(iv) it is soluble in ethyl acetate (Evans, 1979)
(v) it is more active at acid pH than alkaline pH (Pamukcu et al., 1970a; Evans, 1979).

However it is not certain whether it is one toxin that is responsible for the bone marrow aplasia and the resulting haematological syndrome and the wide variety of tumours observed in several experimental animals.

The identity of the bracken carcinogen thus remains the central issue of research on bracken.

1.14 Aims of the Present Study

Of the several candidates nominated as the bracken carcinogen, shikimate and quercetin are deserving of closer consideration because they have produced cancer in experimental animals at certain times and have not at other times. The equivocal nature of the results of experiments on these isolates as well as the public health importance of these isolates make it necessary to re-examine their mechanism of action based on an initiation-promotion hypothesis for the mechanism of action of these two isolates.

It was also necessary to re-examine the factors responsible for the acute toxicity of bracken with a view to fitting both acute and chronic toxicity into an overall mechanism of action for bracken. In this regard the role of the cyanogenic glycoside, prunasin was examined especially as it is known that cyanogenic glycosides cause ataxic syndromes in man and animals.

The indole and kynurenine pathways of tryptophan metabolism were examined in view of the involvement of thiamine as a co-factor in the pathways of tryptophan metabolism.
A comparative approach was adopted, running tests on shikimate, quercetin and bracken in parallel to elucidate the mechanism of action of these compounds.
CHAPTER TWO

THE INITIATING AND PROMOTING PROPERTIES

OF QUERCETIN AND SHIKIMATE
2.1 Introduction

These studies were undertaken to assess quercetin and shikimate as possible initiators and/or promoters in bracken-induced carcinogenesis.

Both compounds had been previously screened for mutagenic, teratogenic and carcinogenic activity. However, the results of the carcinogenicity studies were equivocal and inconsistent giving rise to the view that neither may be complete carcinogens, but instead that they might be contributing to bracken carcinogenicity in initiating and/or promoting roles (Fukushima et al., 1983; Hirose et al., 1983; Kato et al., 1983; Jones et al., 1983).

Although it was possible to demonstrate strong mutagenic activity for quercetin in the Ames test using Salmonella typhimurium TA 98, TA 100, TA 1537, and TA 1538, both with and without activation, attempts to demonstrate its carcinogenicity had shown that it was not a carcinogen, but the work of Pamukcu et al. (1980b) showing that it is capable of producing urinary bladder and intestinal tumours in rats and those of Erturk et al. (1983) suggesting that it is a liver carcinogen tended to ensure renewal of efforts to determine its mode of action with respect to its role in bracken-induced carcinogenicity. Specifically, the reported mutagenic activity of quercetin was of the same order as that of benzo(a)pyrene, a potent carcinogen (Sugimura, 1982). In the light of such mutagenic activity, and equivocal carcinogenicity test results, another hypothesis to explain its role in bracken-induced carcinogenicity was suggested, hence the idea that it might play an initiating (or promoting role) (Fukushima et al., 1983; Hirose et al., 1983; Kato et al., 1983).

Shikimate, although non-mutagenic to Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537 and TA 1538 (Jacobson et al., 1978) was genotoxic in the BHK 21 cell transformation test (Jones et al., 1983). Furthermore, its ability to induce tumours in mice (Evans and Osman, 1974) though not in rats (Hirono et al., 1977) led to the suggestion that it might have another mechanism of action probably a promoting one (Jones et al., 1983) but an initiating role in bracken-induced carcinogenicity cannot be precluded.
2.2 Tumour Initiation and Promotion

One of the characteristics of carcinogenesis is the latent period between contact with a carcinogen and the appearance of a malignant tumour or other neoplastic characteristics. This time lag can be explained on the basis that carcinogenesis is a multistep process and that these steps need time to occur before neoplastic characteristics manifest themselves.

Early investigations identified two steps in the development of epithelial cancers - the initiation phase, which required the application of an initiator and the promotion phase which required the application of an agent which on its own was incapable of producing tumours but could promote the initiated cells to form tumours. This agent was called the promoter (Berenblum and Shubik, 1947).

The process of tumour initiation and promotion was explained as a series of events which require the interaction of two different chemical agents capable of co-operative interaction to produce neoplasia (Berenblum and Shubik, 1947). These events were illustrated by using croton oil as the initiator and benzo(a)pyrene as the promoter with the mouse skin as the experimental model. The various ways in which these could interact are illustrated in Figure 2.1.

Figure 2.1

The Process of Tumour Initiation and Promotion

1. NO TUMOURS
2. TUMOURS
3. TUMOURS
4. NO TUMOURS
5. NO TUMOURS
6. NO TUMOURS

■ = application of initiator
--- = application of promoter

If the initiator alone was applied, or if the initiator was applied after the tumour promoter, no tumours developed. Also, if the tumour
promoter alone was applied or applied at extremely wide intervals after the initiator (in their case, intervals greater than four weeks) no tumours developed.

However, if the tumour promoter was applied after the initiator, no matter how long after the initiator provided that the interval between successive applications was short enough, tumours developed.

2.2.1 Tumour Initiation

An initiating agent is a chemical, physical or biological agent capable of altering the molecular structure of DNA. This alteration may be brought about by covalent binding of the DNA to the agent or its metabolites, distortion of the DNA without covalent binding, complete deletions of whole portions of DNA, scissions at various parts of the DNA chain and/or errors in the repair of any of the above injuries (Pitot and Sirica, 1980). These properties of the initiating agent do not imply that it is the only or absolute requirement for neoplastic transformation.

In studying the initiation process, various cell, organ and animal models have been used. As a result, some characteristics have been observed in some models but not in others. However, only those general features common to most models have been used as the basis for discussing initiation.

The initiating agent exerts its action at the genetic level by inducing alteration in the DNA of the cell. A further step is required to ensure establishment or "fixation" of the initial alteration. This second step is usually in the form of proliferation of initiated cells to create an island or foci or altered cells. These cells differ from the surrounding cells in the possession of properties which render them susceptible to promotion and eventually the formation of neoplasia. It is clear from this brief description of initiation that it is not a single event but a multistep process (Farber, 1980).

There are however certain aspects of the initiation process which remain unclear. These include the essential molecular basis for the induction of the altered cells, the mechanistic role of cell proliferation
in initiation and the physiological and/or metabolic nature of initiated cells that enables them to act as the original progenitors for cancer.

In outline however, the initiation process is irreversible. That is to say, that initiated cells have a "memory" which may be activated at a later time by the appropriate stimulus, usually a promoter, to lead to the formation of tumours. In addition, initiation requires promotion for progression to neoplasia. It is dependent on the stage of the cell cycle and the metabolic machinery of the cell (Pitot, 1981).

2.2.2 Tumour Promotion

A promoting agent is one that alters the expression of the genetic information of the cell. Such an agent does not react directly with the genetic material of the cell but affects its expression by a variety of mechanisms including interaction with cell surface receptors or by an alteration of other cellular components and functions (Pitot and Sirica, 1980).

Various cell, organ and animal models have been used both in vivo and in vitro to study tumour promotion (Hicks, 1983). Although the exact details of each system or model differ in some respects to other systems or models, certain features are common to all of them. Hence, it is postulated that tumour promoters may act by influencing the mutation rate or affecting the repair mechanisms of the cells or by causing de-repression of a regulator gene. It would appear though, that the influence on the mutation rate and the effect on the repair mechanism belong in the realm of tumour initiation, so the more plausible possibility is that tumour promoters may act by de-repression of a regulator gene.

It has however, also been suggested that tumour promoters may act at a cytoplasmic site or at the cell surface. Indeed, these sites appear to have more experimental evidence in their support than the nuclear site as the loci of promoter action (Berenblum and Armuth, 1981).

Whatever the site of promoter action within the cell, its effect is to stimulate a number of initiated cells to develop into neoplastic cells. This may be done by differentially inhibiting surrounding cells thus allowing the
initiated cells to develop preferentially or alternatively by preferential stimulation of the initiated cells over the surrounding cells.

As with the initiation process, there remain areas of insufficient understanding of the promotion process. It is not clear how the promotion stimulus selectively ensures the growth of initiated cells in preference to the surrounding cells. It is also not clear whether the events of the promotion stage are sufficient to provide the momentum that ultimately leads to full neoplastic expression (Farber, 1984).

Although initiation and promotion exist as concurrent processes which follow each other in succession for the realisation of neoplasia, and whilst some compounds have been recognised as being initiating under certain circumstances of dose and route of administration, and others as promoters under various circumstances, there are nevertheless, compounds which on their own are capable of complete carcinogenic activity. These complete carcinogens may mediate their actions via the successive processes of initiation and promotion, but do not require action of other compounds to fulfill their biological effects.

Thus, it is possible to have initiation and promotion as a separate mechanism, different from carcinogenesis induced by a complete carcinogen regimen (Reddy and Fialkow, 1983).

2.3 Markers of Cell Transformation

Various changes may occur in transformed cells in vivo and/or in vitro. These changes have been used as pathognomonic or diagnostic of transformed cells. They may occur at various stages in the process of transformation and with varying intensities.

These changes include alterations in the level or pattern of the enzymes of the cells, morphological changes such as hyperplasia and dysplasia and alterations in the metabolism of the cell resulting in elevations or depressions of the levels of some cell metabolites.

Some of these changes such as hyperplasia may not necessarily lead to neoplasia but may only be a response to external irritating stimuli.
2.3.1 Abnormal Enzymology in Neoplasia

Patients with neoplastic disease show altered profiles of glucose-6-phosphatase and fructose-6-phosphatase (Pitot, 1981). In addition, serum lactate dehydrogenase is elevated in primary hepatoma and serum alkaline phosphatase is elevated in intestinal and bladder neoplasia (Wilkinson, 1976).

Although urinary lactate dehydrogenase had been suggested as a screening test for urinary tract malignancies, the inconsistency of urinary enzyme profiles in such conditions has made them less preferable to serum enzyme assays.

Most of the observations in respect of enzyme changes in neoplasia have been made on human subjects, but similar observations have also been made in experimental animals, in vivo and in cultured mammalian cells in vitro (Wilson et al., 1978; Gadhia and Shah, 1980; Hufnagel et al., 1980; Lehmann et al., 1981).

Changes in enzyme patterns have been associated with the initiation phase of cell transformation and have been demonstrated by the use of enzyme histochemistry in various organs in vivo and in vitro (Farber, 1980), but the definitive evidence of tumour initiating ability is the production of actual tumours in an animal model (Pitot, 1981).

2.3.2 Metabolism in Transformed Cells

In addition to enzymological changes, other activities of transformed cells which may be altered are the rates of plasma membrane ion transport, the synthesis of prostaglandins, polyamines, phospholipids, RNA, DNA, and plasminogen activator, all of which are elevated in transformed cells. In addition, thymidine uptake and the level of large external transformation sensitive (LETS) protein are decreased in transformed cells.

Other changes may occur in the nature of membrane glycoproteins and a delay in early DNA synthesis (Pitot, 1981).
These changes are associated with the promotion phase of cell transformation and though they have been observed mainly in cultured cells in vitro they are also applicable to organ systems in vivo (Wilson et al., 1978; Pitot, 1981).

2.3.3 Morphologic Alterations in Transformed Cells

Increased cell division accompanied by an increase in the mutation rate is an important morphologic characteristic of transformed cells. The increase in cell numbers may be triggered off by a variety of factors but is indicative of the fact that cell division is no longer under ordered control and is an important morphologic manifestation of neoplastic pathology.

The morphologic alterations may be in the form of hyperplasia in which there is an increase in the number of cells, or metaplasia in which cells revert to more primitive forms such as columnar epithelial cells reverting to cuboid or squamous epithelial forms, or they may take the form of anaplasia whereby the inter-relationships of cell organelles are altered.

The alterations (hyperplasia, metaplasia and anaplasia) are considered as part of the post-promotion phase and have been classified as part of tumour progression (Shabad, 1973).

2.4 Tumour Progression

Progression is a series of successive events in which the cell is transformed to attain full malignant status. It refers to a series of events, permanent, irreversible and qualitative which transform the character of a cell until it is malignant (Foulks, 1954; Shabad, 1973; Farber, 1984).

Progression begins with diffuse irregular hyperplasia progressing to focal nodular proliferation during which stage metaplasia and anaplasia may take place leading to adenomas, papillomas and other benign tumours. These may further progress to form malignant tumours (Foulks, 1954, 1958; Shabad, 1973).
Although the process of neoplasia begins from hyperplasia, hyperplasia is not definitely a preneoplastic change. A hyperplastic cell may not revert to normal and sometimes do.

Some aspects of tumour progression remain unclear however. It is not clear how the process is sustained from hyperplasia to neoplasia. It is not certain whether the impetus that sustains the process is endogenous or exogenous. It is also not clear whether the impetus is a "one-hit" phenomenon in which the stimulus once generated is sufficient to achieve neoplasia or a "multi-hit" phenomenon in which continued stimulation is required to achieve neoplastic expression (Farber, 1984).

In recent times, evidence has been emerging of a group of normal cellular genes which may be activated by a variety of external stimuli to produce an abnormal proliferation of cells. These oncogenes, it is thought, may play key or essential roles at each of the major steps in the carcinogenic process. Two or more of these oncogenes may participate in tandem to bring about cell transformation (Farber, 1984).

2.5 Purpose of Study

The roles of quercetin and shikimate in bracken-induced carcinogenesis have been the subject of several studies but it would appear that neither is a complete carcinogen and it has been suggested that they might act as initiators or promoters. To clarify their roles against a background of possible initiation/promotion roles is the subject of this study.

Two sets of studies were carried out to clarify their roles in bracken-induced carcinogenesis. The first of these compared quercetin and shikimate as complete carcinogens to the parent compound, bracken, in a chronic (24 month study), while the second sought to define the properties of both isolates as initiators and/or promoters in a sub-acute (90-day) study.
2.6 The Chronic Study

The aim of this study was to determine the carcinogenicity of quercetin, shikimate and bracken by feeding groups of rats diets containing the test agents and at the end of the study to determine the carcinogenicity of each agent by histopathological examination.

The test agents were fed for 90 days and the rats were then returned to a normal diet for the rest of the study. Although it is the accepted protocol in carcinogenicity bioassays to feed the test substance for the life-span of the test animal, it ought to be borne in mind that this study actually compared the carcinogenic properties of all three compounds so that it is important only to have identical feeding regimes for all the test substances. Therefore, although the test substances were not administered over the life span of the test animals, that need not detract from the merits of this experimental design especially in view of the prohibitive cost of such an experimental design that entails the feeding of some expensive chemicals over an entire life span.

At the end of the study, the experimental animals were autopsied and the histopathological lesions in all the animals assessed to determine whether any of the compounds were capable of inducing tumours or could induce lesions such as hyperplasia and anaplasia or a pre-neoplastic change such as metaplasia especially in the target organs of bracken-induced carcinogenicity, namely the small intestines, the bladder and bone marrow.

2.7 The Sub-Acute Study

The sub-acute study was designed to assess the initiating and promoting properties of quercetin and shikimate by feeding diets containing these bracken isolates to rats in conjunction with known initiators and promoters in a 90-day study. At the end of the study various indices of initiation and promotion were determined.

The bracken carcinogen(s) affect(s) the intestinal tract, the bladder and the bone marrow. In this study emphasis was laid on these three target organs.
The most precise index for tumour initiation is the ultimate production of a tumour but in short-term bioassays, this is not feasible and the use of other indices, such as biochemical lesions may provide an alternative.

Accordingly, by treating the test compounds as "initiators", they were linked up with known promoters and the markers for cell transformation used were those regarded as indices of tumour promotion, but it is assumed that these initiators provide the initial changes that make promotion possible.

Ornithine decarboxylase is an enzyme that forms putrescine, the immediate precursor of spermidine. The polyamines putrescine, spermidine and spermine are found in large quantities in rapidly growing tissues. The decarboxylation of ornithine by ODC is a rate limiting step in the formation of the polyamines and it is thus strategically sited in the metabolism of the polyamines. Therefore in transformed cells or cells undergoing transformation, the level of ODC rises and it is a consistent index of tumour promotion (O'Brien, 1976; Pitot, 1981).

The possible role of plasminogen activators in neoplastic transformation was based on the observations that newly transformed cells in culture showed dramatic increases in plasminogen activator levels (Unkless et al., 1973, 1974). The precise mechanism whereby they are involved in neoplastic transformation is not fully understood but the physiological role of plasminogen activators is to activate plasminogen to plasmin which has a fibrinolytic role in the blood.

It should be emphasised however, that plasminogen activators exist as a wide variety of proteases of which urokinase and streptokinase are the best known, but other tissue and blood activators exist (Christie, 1980).

The effects of quercetin and shikimate on the haematopoietic system were assessed by carrying out various haematological determinations including histopathological examination of the bone marrow at the end of the study. Intermittent blood sampling in the course of a study may yield valuable information in the understanding of a dynamic process such as haematopoiesis, but with rats especially those as small as 55 - 65 g it may also lead to non-compound related pathologies which may obscure genuine toxic effects. The use of satellite groups may be a way round the problem (DHSS, 1982) but it is an added expense.
2.8 Tumour Initiators

The tumour initiators used in the sub-acute studies were those identified in previous studies as having the capability in conjunction with known tumour promoters of inducing characteristics of tumour initiation in various cell, organ and animal models.

2.8.1 N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)

A carcinogen that specifically affects the urinary bladder, BBN, has been used as an initiating agent for the rat at a dose rate of 0.01% (w/v) in the drinking water in conjunction with 5.0% w/w sodium saccharin in the diet as the tumour promoter (Nakanishi et al., 1980). It has also been used as an initiating agent at dose rates of either 5 mg/kg body weight or 50 mg/kg body weight administered intramuscularly weekly to baboons for 2 years in conjunction with Schistosoma haematobium as the promoter (Hicks et al., 1980).

For these studies it was used at a dose rate of 5 mg/kg body weight per rat per week for two weeks by gavage in conjunction with diets containing 5.0% (w/w) saccharin or 1.0% (w/w) shikimate or 1.0% (w/w) quercetin as promoters.

2.8.2 Cyclophosphamide

Cyclophosphamide is toxic to the bone marrow and its metabolite acrolein induces bladder tumours (Hicks, 1980; Ciplea and Mayer, 1981). At an intraperitoneal dose rate of 100 mg/kg body weight per day for five days it induced bone marrow damage in the mouse (Ciplea and Mayer, 1981). At oral dose rates between 0.31 and 2.5 mg/kg body weight per day over a lifetime, it induced between 12 and 37% bladder tumour incidence (Hicks, 1980).

When used at very high doses such as an intraperitoneal dose rate of 100 mg/kg body weight per month for eighteen months or a single intraperitoneal dose of 200 mg/kg body weight in conjunction with methyl nitrosourea (MNU), no tumours were produced in the rat and it was suggested
that at such high dose rates, cyclophosphamide became cytotoxic, killing the urothelial cells rather than inducing a heritable change in the target cells (Hicks, 1980).

A lower dose rate similar to that adopted by Arai et al. (1977) was considered more suitable. They used a dose rate of 10 mg/kg body weight twice a week in male rats for forty weeks and failed to induce any tumours, indicating that at this dose, cyclophosphamide was non-carcinogenic. At the same dose rate it was capable of promoting FANFT-initiated cells to produce tumours.

For these studies, cyclophosphamide was used at an intraperitoneal dose rate of 10 mg/kg body weight per rat per week for two weeks as the initiating agent in conjunction with the same compound and dose rate, 1.0% (w/w) a quercetin-containing diet or a 1.0% (w/w) shikimate-containing diet, all as promoters, for the studies on the haematopoietic system. The ability of cyclophosphamide to act as a bladder carcinogen also made it important to assess its effects on the bladder.

2.8.3 N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

MNNG is carcinogenic to the alimentary tract. It has been used as an initiating agent at an intrarectal dose rate of 2.5 mg per rat, twice a week for two weeks in conjunction with sodium lithocholate, during which colonic tumours were produced (Reddy and Watanabe, 1979).

When administered at an oral dose rate of 83 mg/L in the water in conjunction with 2.0% (w/w) taurocholic acid in the diet tumours were produced in the stomach and duodenum (Salmon et al., 1984).

When administered at an oral dose rate between 100 ug/ml and 167 ug/ml in the drinking water in conjunction with the bacterium, pseudomonas aeruginosa as promoter, gastric and small intestinal tumours were produced (Morishita and Shimizu, 1983).

In these studies MNNG was used at an oral dose rate of 7.5 mg/kg body weight per rat per week for two weeks, with 1.0% (w/w) lithocholate, 1.0% (w/w) quercetin or 1.0% (w/w) shikimate as the promoters.
2.9 Tumour Promoters

The tumour promoters used in the study were those described in various previous studies as capable of tumour promoting activity.

2.9.1 Saccharin

When fed to rats as a 5.0% (w/w) diet for a period of 100 weeks after 0.2% (w/w) FANFT diet fed for four weeks, saccharin was shown to be capable of tumour promotion in the rat urinary bladder, producing papillomas and carcinomas (Fukushima et al., 1981). It has also been administered in the diet (5% w/w) in conjunction with BBN as the initiator to induce urinary bladder tumours in Wistar rats (Nakanishi et al., 1980).

In these studies it was used as a 5.0% (w/w) diet as a tumour promoter in conjunction with 5 mg/kg BBN, 1.0% (w/w) shikimate diet and 1.0% (w/w) quercetin diet as initiators.

2.9.2 Lithocholic Acid

When administered to rats at an intra-rectal dose rate of 20 mg per rat thrice weekly for 46 weeks after initiation by MNNG, lithocholic acid had a promoting action on colon carcinogenesis (Reddy and Watanabe, 1979).

In these studies it was administered in the diet (1% w/w) in conjunction with MNNG, 1.0% (w/w) quercetin diet, and 1.0% (w/w) shikimate diet as initiators.

2.9.3 Cyclophosphamide

Cyclophosphamide has been used as a promoting agent at an oral dose rate of 10 mg/kg body weight twice a week, in conjunction with FANFT as the initiating agent (Arai et al., 1977).
In the initiation-promotion studies however, it was used at an intraperitoneal dose rate of 10 mg/kg body weight once a week because it is cytotoxic at high doses and its toxicity via the oral route is more rapid in onset than via the intraperitoneal route.

In these studies it was used as a haematotoxic agent but its toxicity to the urinary bladder has also been considered.

2.10 Materials and Methods

2.10.1 Experimental Animals

Male Wistar-albino (W/A) rats (University of Surrey, Animal Unit) were assigned twenty per group for the chronic study and five per group for the sub-acute study so that each group weighed on average 60.0 ± 5.0 gm.

For both studies the rats were housed five per cage in plastic rat cages on a bedding of wood chips. The cages had dimensions of 56.25 x 33.75 x 15.0 cm with metal grill tops. For the chronic studies, the rats were rehoused three per cage after six months and two per cage after eighteen months.

2.10.2 Feedstuffs and Diets

Bracken was collected from the areas in and around Worplesdon, Surrey, during July and August, dried at 100°C in a dry heat oven (Department of Chemical Engineering, University of Surrey), powdered with a mortar and pestle, sieved and incorporated as 10% (w/w) of the diet for both studies.

Quercetin and shikimate (Sigma Chemical Company Ltd., Poole, Dorset, England) were made up as 1.0% (w/w) diets with powdered rodent diet for both chronic and sub-acute studies.

The control rats received powdered rodent diet only (appendix IV). All the diets were presented in steel feeding pots placed inside the cages. In all groups, food and water were available ad libitum.
None of the animals were given thiamine supplementation to their diets because of the added cost of implementing adequate control groups.

The strategy of the chronic study was to feed the test compounds in the diet for 90 days and return the rats to control diets for the remainder of their lives (i.e. until two years) when the study was terminated.

The sub-acute study was carried out according to the dosing regime shown in Table 2.1.

2.10.3 Chemicals and Reagents

The chemicals and reagents were of analytical reagent grade unless otherwise specified.

2.10.4 Clinical Observations

The rats were observed daily for signs of illness and weighed weekly.

2.10.5 Sample Collection

After 90 days, the rats on the sub-acute study were lightly anaesthetised with anaesthetic ether (May and Baker Ltd., Manchester, England) and terminally bled out via the posterior vena cava. Blood for haematological assays was collected in tubes containing EDTA as anticoagulant. Tissues for histopathology were collected in 10% buffered formalin and tissues for PA and ODC assays were washed in physiological saline and placed in ice cold buffer (2.10.6 and 2.10.8).

At the termination of the chronic study, the rats were anaesthetised as described above, bled out terminally, necropsied and the various tissues collected in 10% buffered neutral formalin for histopathological examination.
### Table 2.1

**Dosing Regime to Study the Initiating and Promoting Properties of Quercetin and Shikimate**

<table>
<thead>
<tr>
<th>TIME (weeks)</th>
<th>INITIATION PHASE</th>
<th>PROMOTION PHASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10% w/w bracken diet</td>
<td>5.0% w/w saccharin diet</td>
</tr>
<tr>
<td>2</td>
<td>1% w/w quercetin diet</td>
<td>10 mg/kg body weight cyclophosphamide per week intraperitoneally</td>
</tr>
<tr>
<td>3</td>
<td>1% w/w shikimate diet</td>
<td>10 mg/kg body weight cyclophosphamide per week intraperitoneally</td>
</tr>
<tr>
<td>13</td>
<td>5.0% w/w saccharin diet</td>
<td>1% w/w lithocholate diet</td>
</tr>
<tr>
<td></td>
<td>10 mg/kg body weight cyclophosphamide per week intraperitoneally</td>
<td>1% w/w lithocholate diet</td>
</tr>
<tr>
<td></td>
<td>1% w/w quercetin diet</td>
<td>5.0% w/w saccharin diet</td>
</tr>
<tr>
<td></td>
<td>5 mg/kg body weight BBN per week by gavage</td>
<td>1% w/w quercetin diet</td>
</tr>
<tr>
<td></td>
<td>1% w/w shikimate diet</td>
<td>1% w/w lithocholate diet</td>
</tr>
<tr>
<td></td>
<td>5 mg/kg body weight BBN per week by gavage</td>
<td>1% w/w shikimate diet</td>
</tr>
</tbody>
</table>
Table 2.1 (Continued)

**Dosing Regime to Study the Initiating and Promoting Properties of Quercetin and Shikimate**

<table>
<thead>
<tr>
<th>TIME (weeks)</th>
<th>0</th>
<th>2</th>
<th>3</th>
<th>13</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>INITIATION PHASE</strong></th>
<th><strong>PROMOTION PHASE</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 mg/kg body weight MNNG per week by gavage</td>
<td>1% w/w quercetin diet</td>
</tr>
<tr>
<td>7.5 mg/kg body weight MNNG per week by gavage</td>
<td>1% w/w shikimate diet</td>
</tr>
<tr>
<td>10 mg/kg body weight cyclophosphamide per week i/p</td>
<td>1% w/w quercetin diet</td>
</tr>
<tr>
<td>10 mg/kg body weight cyclophosphamide per week i/p</td>
<td>1% w/w shikimate diet</td>
</tr>
<tr>
<td>10 mg/kg body weight cyclophosphamide per week i/p</td>
<td>normal rodent diet</td>
</tr>
<tr>
<td>5 mg/kg body weight BBN per week by gavage</td>
<td>normal rodent diet</td>
</tr>
<tr>
<td>7.5 mg/kg body weight MNNG per week by gavage</td>
<td>normal rodent diet</td>
</tr>
<tr>
<td>7.5 mg/kg body weight MNNG per week by gavage</td>
<td>1% w/w lithocholate diet</td>
</tr>
<tr>
<td>10 mg/kg body weight cyclophosphamide per week i/p</td>
<td>5 mg/kg body weight BBN per week by gavage</td>
</tr>
<tr>
<td>5 mg/kg body weight BBN per week by gavage</td>
<td>5.0% w/w saccharin diet</td>
</tr>
</tbody>
</table>

**Key**

a = control diet only  
 b = control diet plus distilled water by gavage, weekly  
 c = control diet plus distilled water intraperitoneally, weekly  

BBN = N-butyl-N-(4-hydroxybutyl) nitrosamine  
MNNG = N-methyl-N'-nitro-N-nitrosoguanidine  
i/p = intraperitoneally

BBN, MNNG, cyclophosphamide and sodium lithocholate were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England.
2.10.6 Ornithine Decarboxylase Assay

Ornithine decarboxylase was determined as described by Russell and Snyder (1968) with the modifications as applied by Hosaka et al. (1983).

The tissue was excised, washed in physiological saline and homogenised in 2 volumes of an ice-cold solution of 50 mM pyridoxal phosphate buffer, pH 7.4, containing 0.1 mM pyridoxal phosphate (Sigma Chemical Co. Ltd., Poole, Dorset, England) and 0.1 mM EDTA (Sigma Chemical Co. Ltd., Poole, Dorset, England) using a Potter-Elvehjem glass teflon homogeniser (three return strokes). The homogenate centrifuged at 2°C in an MSE Superspeed 65 Centrifuge (MSE Scientific Instruments Ltd., Sussex, England) at 30,000 g for 30 minutes. From the supernatant 0.5 ml aliquots were added to 1.0 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 0.2 mM pyridoxal phosphate 1.0 mM EDTA, 1.0 mM dithiothreitol (Sigma Chemical Co. Ltd., Poole, Dorset, England) and 0.5 uCi of D,L-ornithine 1-14C hydrochloride (58 mCi/mmol), (Amersham International PLC, Buckinghamshire, England) in 0.5 mM L-ornithine (Sigma Chemical Co. Ltd., Poole, Dorset, England). The reaction was carried out in flasks stoppered with self-sealing rubber caps fitted with polyethylene hanging centre wells (Kontes Scientific Glassware, Vineland, New Jersey, USA). The centre wells contained 0.3 ml of a 2 : 1 (v/v) ethanolamine : methoxyethanol mixture (May and Baker Ltd., Manchester, England), to absorb the 14CO2 released from ornithine hydrochloride by the action of ornithine decarboxylase.

The flask was incubated at 37°C for 30 minutes at the end of which the reaction was terminated with 0.8 ml of 50% (w/v) citric acid Chemicals Ltd., Poole, Dorset, England). The flask was left for 12 hours still stoppered to ensure complete absorption of the 14CO2.

The ethanolamine : methoxyethanol mixture was transferred to 10.0 ml of Optiphase 'Safe' scintillant (Manufactured for LKB, Wallac, Turku, Finland by Fisons PLC, Loughborough, England). The amount of radioactivity was determined using an LKB 1217 Rackbeta Liquid Scintillation Counter (LKB, Wallac, Turku, Finland). The counting efficiency of each sample was determined by adding an internal standard (glycerol tril(14C)stearate) with an activity of 102,500 dpm, accurate to within 0.2% of stated activity (LKB, Wallac, Turku, Finland), to each test sample and recounting as described above. The efficiency of counting of each sample was
(Count rate of sample + internal standard) - Count rate of sample
d.p.m. of standard

This was multiplied by 100 to give percentage efficiency which in the
test sample ranged between 88 - 95%. ODC activity was expressed as pmole
$^{14}$CO$_2$/30 min/mg protein.

2.10.7 Determination of Protein Content

Samples of the supernatant were diluted 1 : 50 with distilled water and
duplicate 1.0 ml aliquots of the diluted supernatant were added to test
tubes containing 5.0 ml copper reagent made up of 1.0 ml 1% (w/v) CuSO$_4$, 1.0
ml of 2% (w/v) sodium potassium tartrate and 100 ml of 2% (w/v) sodium
carbonate in 0.1 M sodium hydroxide. After 10 minutes, 0.5 ml Polin and
Ciocalteau's Phenol Reagent (Sigma Chemical Co. Ltd., Poole, Dorset,
England) diluted 1 : 1 with distilled water was added and mixed rapidly.
After 30 minutes the absorbance was determined at 750 nm in a Cecil CE 292
digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England)
using a blank which contained distilled water instead of the supernatant
(Lowry et al., 1951).

The protein standard curve was prepared with serial dilutions from a
stock solution of 20 mg/ml bovine serum albumin (BSA). The bovine serum
albumin was treated as the supernatant and the assay was linear over the
range 0 - 40 ug/ml. The standard curve was obtained after linear regression
analysis using a linear regression package based on the University of Surrey
Prime Computer System.

2.10.8 Plasminogen Activator Assay

The determination of plasminogen activator was based on the amidolytic
assay method using a chromogenic substrate as used by Whur et al. (1980)
with some modifications.

The amidolytic assay is based on the principle that in the presence of
excess urokinase (a plasminogen activator) a complex is formed which
catalyses the formation of p-nitroaniline from H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride (S-2251) (Kabi Diagnostica, Stockholm, Sweden). If the situation is reversed so that there is an excess of plasminogen the reaction will also proceed but will then be dependent on the level of urokinase.

\[
\text{H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride} \\
(\text{chromogenic substrate S-2251}) \\
+ \\
\text{Plasminogen} \\
+ \\
\text{Urokinase} \\
\text{H}_2\text{O} \\
= \text{Urokinase Complex} \\
\text{(activator)} \\
\text{H-D-valyl-leucyl-lysine-OH} + \text{p-nitroanilide (yellow)}
\]

The absorbance of the p-nitroanilide at 405 nm is proportional to the activity of urokinase provided that plasminogen is in excess.

The standard curve was prepared by using various concentrations of urokinase (Sigma Chemical Co. Ltd., Poole, Dorset, England) in 20 ul aliquots made up in Hanks Buffered Saline Solution (HBSS). The 20 ul aliquot of urokinase was added to tubes containing 200 ul of 3.0 mM, S-2251, 400 ul HBSS, 100 ul of 1 CU/ml plasminogen (Kabi Diagnostics, Sweden). S-2251 and plasminogen were made up in HBSS. It was incubated for 10 minutes at 37°C and the reaction terminated with 100 ul of 50% acetic acid (BDH Chemicals Ltd., Poole, Dorset, England). The absorbance was determined at 405 nm in a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England). The blank was set up as the tests except that the reaction was stopped immediately (time = zero). The assay was linear over the range 0.345 x 10^3 to 27.75 x 10^3 International Units per ml. The standard curve was the computer generated best fit line produced after linear regression analysis package based on the University of Surrey, Prime Computer System.

The test tissues were homogenised in 10 volumes of ice-cold HBSS in a motor driven Potter-Elvehjem glass teflon homogeniser (three return strokes). The homogenate was centrifuged at 20,000 g for 20 minutes at 4°C in a Beckman J2-21 Centrifuge (Beckman-RIIC Ltd., Buckinghamshire, England).
From the supernatant, 20 ul aliquots were used for the determination of plasminogen activator activity, described above for the standard curve. The blanks contained the test supernatant but the reaction was stopped immediately with acetic acid.

The protein content of the supernatant was determined as described in 2.10.7. Plasminogen activator activity determined as urokinase was expressed as International Units (IU) per mg protein.

2.10.9 Haemoglobin Assay

Haemoglobin was determined by the cyanmethaemoglobin using Drabkin’s Reagent which was 200 mg potassium ferricyanide, 50 mg potassium cyanide and 140 mg potassium dihydrogen phosphate up to 1 L with distilled water.

The standard curve was prepared using commercially available haemoglobin standards containing 3.0 g %, 11.5 g % and 18.0 g % of haemoglobin (Diagnostic Reagents Ltd., Thame, Oxon; England) using Drabkin’s reagent as the blank. The absorbance at 540 nm was determined in a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England). The standard curve was the computer generated best fit line obtained after linear regression analysis using a linear regression analysis package based on the University of Surrey Prime Computer System.

The test samples in EDTA were made up as 1 : 200 solutions with Drabkin’s Reagent and the absorbance determined at 540 nm in a Cecil CE 292 digital UV spectrophotometer. The haemoglobin content was determined from the standard curve and expressed as g %.

2.10.10 Determination of Packed Cell Volume (PCV)

Thin capillary tubes were filled with blood containing EDTA and sealed at one end with plasticine and centrifuged in a microhaematocrit centrifuge (MSE Scientific Instruments Ltd., Sussex, England). The percentage haematocrit was determined in a microhaematocrit reader (MSE Scientific Instruments Ltd., Sussex, England). Each blood sample was analysed in triplicate.
2.10.11 Total Erythrocyte and Leucocyte Counts

A Coulter Electronic Counter, Model Dn (Coulter Electronics Ltd., Coldharbour Lane, Harpenden, Herts., England) was used for counting of erythrocyte and leucocytes in blood samples. The window setting for each set of blood samples was pre-determined with control blood samples.

2.10.12 Derived Haematological Indices

The Mean Cell Volume (MCV) was derived as follows

\[ \text{MCV} = \frac{\text{Packed Cell Volume (\%)} \times 10}{\text{Total Erythrocyte Count (million/ul)}} \] (femtolitres)

The Mean Cell Haemoglobin was derived from the formula

\[ \text{MCH} = \frac{\text{Haemoglobin (g/dl)} \times 10}{\text{Total Erythrocyte Count (million/ul)}} \] (picograms)

The Mean Corpuscular Haemoglobin Concentration (MCHC) was calculated as follows

\[ \text{MCHC} = \frac{\text{Haemoglobin (g/dl)} \times 100}{\text{Packed Cell Volume (\%)}} \] (%)

These indices are derived for quality control purposes. An MCV range of 53-69 femtolitres, an MCH range of 17-22 picograms and an MCHC range of 29-34% being regarded as within normal limits.

2.10.13 Platelet Counts

The manual method of Austen and Rhymes (1975) was used for the determination of platelet numbers in blood. Blood was diluted 1:100 with freshly prepared and filtered 1% (w/v) ammonium oxalate (BDH Chemicals Ltd., Poole, Dorset, England). The diluted blood sample was introduced into an Improved Neubauer Counting Chamber making sure that there were no air bubble when it was covered with a coverslip.
The Counting Chamber was left on a wet surface consisting of paper towels soaked in water. After ten minutes, the platelets seen as highly refractile bodies were counted in an area 1/5 sq.mm. (made up of sixteen small squares) in a phase contrast microscope with a magnification of x 400.

The ammonium oxalate lyses the erythrocytes leaving the leucocytes and platelets.

The platelet count per cubic millimetre = total count x 5000

2.10.14 Differential Leucocyte Counts

Thin blood films were made on glass slides, identified with a diamond marker on the same side as the smear, air dried and fixed in analytical grade methanol (May and Baker Ltd., Manchester, England) for 10 minutes.

The slides were stained for 5 minutes in May-Grunwald stain which was made up of equal parts of the commercial stain (Raymond A. Lamb, Sunbeam Road, London, England), and distilled water buffered to pH 6.5 with Sorensen's buffer (30 ml of 9.465 g/L disodium hydrogen phosphate and 70 ml of 9.07 g/L potassium dihydrogen phosphate, pH 6.5) (BDH Chemicals Ltd., Poole, Dorset, England).

The slides were drained of May-Grunwald stain and stained in stain which was made up of one part of the commercial stain (Raymond A. Lamb, Sunbeam Road, London, England), and nine parts of distilled water buffered with Sorensen's buffer pH 6.5. The slides were stained in Giemsa stain for 10 minutes.

The slides were drained of Giemsa stain, dried, and examined under oil immersion with a magnification of x 400.

One hundred leucocytes were counted and classified by moving the slide across the lens in a "zig-zag" fashion. The leucocytes were classified as eosinophils, neutrophils, basophils, monocytes and lymphocytes.

The absolute numbers of each type of leucocyte was calculated from the total leucocyte count.
2.10.15 Reticulocyte Counts and Heinz Body Detection

Three drops of blood collected in EDTA were mixed in an LP3 tube (Luckham Ltd., Burgess Hill, Sussex, England) with 4 drops of 1% Brilliant Cresyl Blue (Raymond A. Lamb, Sunbeam Road, London, England) in citrate saline. The citrate saline was made up of 1 part of 3% (w/v) aqueous trisodium citrate (BDH Chemicals Ltd., Poole, Dorset, England) and 4 parts of 0.9% (w/v) sodium chloride (BDH Chemicals Ltd., Poole, Dorset, England). The LP3 tubes containing blood and stain were capped, and incubated for 2 minutes at 37°C. Each tube was gently agitated and thin smears were made on plain glass slides, air dried and identified with a glass diamond marker. The films were examined at a magnification of x 400 under oil immersion. The number of reticulocytes in 20 fields was determined ensuring that at least 100 reticulocytes were counted. The number of erythrocytes in four fields, the 5th, 10th, 15th and 20th fields was also determined and percentage reticulocytes in 20 fields was determined by:

\[
\text{number of reticulocytes counted in 20 fields} \times \frac{100}{5 \times \text{number of erythrocytes in four fields}}
\]

The absolute numbers of reticulocytes were obtained form the total erythrocyte counts.

2.10.16 Gross and Histopathological Examination

At the termination of the experiments, the rats were ether anaesthetised and bled to death via the posterior vena cava and all the major organs examined for gross pathological lesions. Where these were found they were noted, and tumours were measured as to diameter.

Tissues for histopathological examination were thinly cut and fixed in 10% buffered neutral formalin and after three weeks were processed for histopathological examination as described by Bloom and Fawcett (1970). The slides were stained with haematoxylin and eosin and examined under a light microscope.
2.11 Results

The results of the chronic study are represented by the mean values of 20 rats ± standard error of the mean (SEM), while for the sub-acute study they are the mean values of 5 rats ± standard error of the mean (SEM).

2.11.1 Statistical Analysis

The Students' t-test was used to evaluate the data and compare the various groups.

Chronic Studies

2.11.2 Weight Changes

The control, quercetin- and shikimate-fed rats gained weight steadily throughout the study, while the bracken-fed rats had a slower rate of weight gain which in the period up to week 4 differed significantly (p <0.005) and after week 4 (p <0.001) from the control (Figure 2.2).

2.11.3 Clinical Observations

Bracken-fed rats were visibly listless from day 14 on the bracken diet. They had starring hair coats and by day 22 tended to tremble slightly. By day 26, four of these rats developed ataxia and by day 27, three more developed this clinical sign. Their reflexes were dull, they had an unsteady gait as well as torticollis.

On day 28, one of the bracken-fed rats died and was necropsied. To avoid the autolytic changes that accompany such sudden deaths, all the ataxic rats were ether anaesthetised, terminally exsanguinated and autopsied. For comparative purposes, five rats from the control group were autopsied at this time.
Weights are the mean of twenty rats per group.

(4) The bracken-fed rats were statistically significantly different from the control, p < 0.005 up to week 4 and p < 0.001 up to the termination of the study.
At 8.5 months, one of the bracken-fed rats was found dead and was necropsied. For comparative purposes, one each from the control, quercetin-fed and shikimate-fed rats were also autopsied after ether anaesthesia and exsanguination via the posterior vena cava.

In order to avoid further sudden deaths and the difficulties which could be imposed by autolytic changes that would accompany them, the remaining twelve bracken-fed rats were electively ether anaesthesised, bled out and necropsied at 8.5 months along with four control rats.

At 24 months, nineteen rats each from the quercetin- and shikimate-fed groups of rats and ten from the control group were autopsied.

During the course of the study, none of the rats in the control group or in the quercetin- and shikimate-fed groups showed any clinical signs of illness.

2.11.4 Gross Pathology

The ataxic bracken-fed rats necropsied at week 4 revealed loss of subcutaneous fat.

The bracken-fed rat that died suddenly at 8.5 months had an ileal intussuscception with a portion of the ileum telescoping over an ileal tumour into the succeeding part up to a distance of 1.5 cm. Other polypous tumours were found in the ileum with diameters, 0.2 - 0.5 cm.

Of the twelve bracken-fed rats electively autopsied at 8 months, eleven had tumours of diameters 0.2 - 1.0 cm in the jejunum and ileum (Plates III and IV).

There was a roughening of the cortical surface of the kidneys in all rats autopsied from 8.5 months on and increasing with age.
PLATE III - Portion of ileum of male Wistar-albino rat fed 10% (w/w) bracken diet showing an ileal adenoma at 8.5 months.

PLATE IV - Cross section of tubulous adenoma induced in rats fed 10% (w/w) bracken diet (x 4) H & E.
2.11.5 Histopathology

The bracken-fed rats autopsied at week 4 had a loss of red pulp in the spleen, with eosinophil infiltration of the sinusoids and increasing numbers of macrophages. The liver had a slight dilation of the sinusoids while the kidneys had a slight thickening of the serosal surface of the Bowman's Capsule. The predominant impression in the bone marrow was of megakaryocyte infiltration.

The bracken-fed rat that died of intususception had an ileal tumour, but its histopathology was obscured by autolytic changes that had set in before necropsy.

The electively necropsied bracken-fed rats revealed ileal tumours which at histopathology were identified as tubulous adenomas (Plates V and VI). These tumours were characterised by increased mitoses in the epithelial cells, mild to severe epithelial dysplasia but in all cases they were sharply circumscribed and the muscularis mucosa was not penetrated.

There was increased mitoses in the hepatocytes as well as a widening of the sinusoids. In the spleen, the red pulp was diminished, there was deposition of golden brown pigment and the presence of ceroid, a finding also present in the control. In the kidneys of the bracken, control, quercetin and shikimate rats autopsied at 8.5 months, there was thickening of the serosal surface of the Bowman's Capsule and there were proteinaceous casts in the collecting ducts.

In the quercetin, shikimate and control rats autopsied at 24 months, the predominant impression in the spleen was of deposition of ceroid, the red pulp was more diffuse and less compact and apparently diminished, there was golden-brown pigment both intra- and extra-corporuscular and an increase in the number of macrophages. In the kidney, tubular dilation was evident as well as tubular atrophy, there was deposition of protein casts and foci of mononuclear cells. In the papilla there was moderate vacuolation.
PLATE V - Section of tubulous adenoma from rat fed 10% (w/w) bracken diet, showing non-invasion of the muscularis mucosa by the benign tumour (x 50) H & E.

PLATE VI - Ileal tubulous adenoma from rat fed 10% (w/w) bracken diet, showing eosinophilic paneth cells and mitotic figures (x 313) H & E.
Sub-acute Studies

2.11.6 Weight Changes

The bracken-fed rats had a slower rate of weight gain than the control group (\( p < 0.001 \)) during the experimental period. The other test groups did not differ significantly from the control group (Figure 2.3).

2.11.7 Clinical Observations

The bracken-fed rats were listless and had piloerection. There were no clinical signs of illness in the rats in the other experimental groups.

2.11.8 Gross Pathology

The bracken-fed rats were cachetic, had little subcutaneous fat, and rough hairy coat.

2.11.9 Histopathology

The liver of the bracken-fed rats revealed eosinophilic hepatocytes with cytoplasmic and nuclear swelling in the centrilobular region accompanied by an increase in the number of mitotic figures and a widening of the sinusoids. The rats treated with cyclophosphamide over 11 and 13 weeks respectively (i.e. as promoter and initiator-promoter) had occasional single cell necrosis and increased mitosis.

The spleen of the quercetin, bracken, shikimate, cyclophosphamide (promoter and initiator-promoter), cyclophosphamide plus quercetin, cyclophosphamide plus shikimate, shikimate plus cyclophosphamide, and quercetin plus cyclophosphamide groups revealed diminished red pulp and extrafollicular lymphoid hyperplasia in the central and littoral zones. Additionally, in these as well as in the control and saccharin groups there was deposition of golden-brown pigment which in the quercetin, bracken and shikimate groups was deposited both intra- and extra-cellular with respect to the increased numbers of macrophages in these groups and in the cyclo-
Figure 2.3 Weight Gains of Rats in 90-Day Initiation-Promotion Study

(1) Control diet
(2) Various initiators and promoters
(3) $10^7$ (w/w) bracken diet

(1) Weights are the mean of fifteen rats from three groups (i.e. five per group).

(2) Weights are the mean of 115 rats from twenty-three groups (i.e. five per group).

(3) Weights are the mean of five rats.

Bracken-fed group was significantly different from the control groups, $p < 0.001$ throughout the study period.
phosphamide (initiator-promoter and promoter groups) cyclophosphamide plus quercetin, cyclophosphamide plus shikimate, shikimate plus cyclophosphamide and quercetin plus cyclophosphamide groups. There was increased splenic congestion in the saccharin group, while in the bracken group there was an increased number of mitotic figures, and foci of necrobiotic cells and siderophages, the latter being found in the quercetin group also. In the bracken group there was infiltration of eosinophils into the sinusoids.

The kidneys of the control, quercetin, bracken, dibutyl nitrosamine and cyclophosphamide (initiator-promoter) groups revealed congestion of the vasa recta and in the quercetin group there was additionally focal tubular metachromasia, and in the shikimate group, epithelial desquamation of the collecting ducts.

The bone marrow of the shikimate and quercetin groups revealed slight fatty infiltration and the replacement of the marrow by lipocytes. The bracken group revealed an advanced but moderate stage of this lesion while the cyclophosphamide (promoter and initiator-promoter) groups represented a more severe stage of the lesion with increasing replacement of the marrow by lipocytes. The cyclophosphamide plus quercetin, cyclophosphamide plus shikimate and shikimate plus cyclophosphamide groups revealed a near total replacement of the marrow by lipocytes.
Table 2.2 Reproducibility\(^a\) and sensitivity\(^b\) of the analytical methods employed.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Method</th>
<th>Reproducibility (%)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine</td>
<td>(^{14}\text{C} - \text{Liquid}) Scintillation Counting</td>
<td>Mean: 46, Range: 8-93</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>Protein</td>
<td>Folin and Ciocalteau's Phenol Reagent</td>
<td>Mean: 12, Range: 6-18</td>
<td>1.2 ug/ml</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Chromogenic Substrate Assay using S-2251</td>
<td>Mean: 52, Range: 2-149</td>
<td>345 IU/ml</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>Drabkin's Reagent</td>
<td>Mean: 14, Range: 2-26</td>
<td>3 g%</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>Microhaematocrit Centrifuge</td>
<td>Mean: 6, Range: 2-11</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>Total Erythrocytes</td>
<td>Coulter Counter Model Dn</td>
<td>Mean: 9, Range: 4-17</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>Total Leucocytes</td>
<td>Coulter Counter Model Dn</td>
<td>Mean: 24, Range: 4-64</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>Total Platelets</td>
<td>Ammonium oxalate method of Austen and Rhymes (1975)</td>
<td>Mean: 24, Range: 5-66</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>Total Reticulocytes</td>
<td>Microscopic evaluation of thin smears</td>
<td>Mean: 0.4, Range: 0.2-0.8</td>
<td>Not Applicable</td>
</tr>
</tbody>
</table>

continued
Table 2.2 Reproducibility\(^a\) and sensitivity\(^b\) of the analytical methods employed (continued).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Method</th>
<th>Reproducibility (%)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Cell Volume</td>
<td>PCV (\times 10 = (\text{fm})) RBC</td>
<td>Mean: 4.1</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range: 1.5-7.9</td>
<td>Applicable</td>
</tr>
<tr>
<td>Mean cell Haemoglobin</td>
<td>Haemoglobin (\times 10 \text{ (pg)}) RBC</td>
<td>Mean: 4.9</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range: 1.7-8.2</td>
<td>Applicable</td>
</tr>
<tr>
<td>Mean Corpuscular</td>
<td>Haemoglobin (\times 100 \text{ (%)}) PCV</td>
<td>Mean: 7.0</td>
<td>Not</td>
</tr>
<tr>
<td>Haemoglobin Concentration</td>
<td></td>
<td>Range: 1.7-26.4</td>
<td>Applicable</td>
</tr>
<tr>
<td>Eosniophils</td>
<td>Microscopic evaluation of thin smears</td>
<td>Mean: 49</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range: 34-70</td>
<td>Applicable</td>
</tr>
<tr>
<td>Basophils</td>
<td>Microscopic evaluation of thin smears</td>
<td>Mean: 104</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range: 71-220</td>
<td>Applicable</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Microscopic evaluation of thin smears</td>
<td>Mean: 52</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range: 9-102</td>
<td>Applicable</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Microscopic evaluation of thin smears</td>
<td>Mean: 59</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range: 21-107</td>
<td>Applicable</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Microscopic evaluation of thin smears</td>
<td>Mean: 5</td>
<td>Not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range: 3-14</td>
<td>Applicable</td>
</tr>
</tbody>
</table>

\(a\) - The reproducibility of each method was assessed by determining the coefficient of variation by the formula (standard deviation/mean) \(\times 100\) to give a percentage value which is indicative of how easily reproducible repeated analysis with the method is. The smaller the value, the better the method used.

\(b\) - Sensitivity refers to the smallest quantity of the metabolite or substance that may be detected with the method. The smaller the value, the more sensitive the method is.
### 2.11.10 Ornithine Decarboxylase Activity

The shikimate-fed rats had significantly elevated levels of ODC activity in the liver, intestine, spleen and kidney compared to the control, but the quercetin and bracken groups did not differ significantly from the controls in this regard (Table 2.3).

**Table 2.3 Ornithine Decarboxylase (ODC) Activity at day 90, in Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets in the Sub-Acute Study**

<table>
<thead>
<tr>
<th>Group</th>
<th>Bladder (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
<th>Liver</th>
<th>Intestine</th>
<th>Spleen</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>151±22</td>
<td>3±1</td>
<td>14±2</td>
<td>13±2</td>
<td>30±6</td>
</tr>
<tr>
<td>Quercetin</td>
<td>125±17</td>
<td>3±1</td>
<td>10±2</td>
<td>15±1</td>
<td>32±8</td>
</tr>
<tr>
<td>Bracken</td>
<td>137±14</td>
<td>3±0</td>
<td>18±1</td>
<td>15±1</td>
<td>10±2</td>
</tr>
<tr>
<td>Shikimate</td>
<td>1026±436</td>
<td>119±48*</td>
<td>560±80*</td>
<td>100±5*</td>
<td>87±3*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.

* - value significantly higher than control (p < 0.05)

(Using the Student's t-test)

In the initiation studies the quercetin plus cyclophosphamide, shikimate plus cyclophosphamide, the quercetin plus saccharin, shikimate plus saccharin, quercetin plus lithocholate and shikimate plus lithocholate treatments enhanced the levels of ornithine decarboxylase in the tissues examined although it was not always consistent (Tables 2.4, 2.5 and 2.6).
### Table 2.4 Ornithine Decarboxylase (ODC) Activity at day 90, in the Bladder and Liver of Rats in the Initiation Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initiator</td>
</tr>
<tr>
<td>-</td>
<td>Cyclophosphamide$^a$</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>Shikimate</td>
<td>Cyclophosphamide</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

- control group

* - value significantly higher than control (p < 0.05)

+ - value significantly lower than control (p < 0.05)

(Using the Student’s t-test)

### Table 2.5 Ornithine Decarboxylase (ODC) Activity at day 90, in the Bladder and Liver of Rats in the Initiation Study on Quercetin and Shikimate using BBN and Saccharin

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initiator</td>
</tr>
<tr>
<td>-</td>
<td>Saccharin$^a$</td>
</tr>
<tr>
<td>BBN</td>
<td>Saccharin</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Saccharin</td>
</tr>
<tr>
<td>Shikimate</td>
<td>Saccharin</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

- control group

* - value significantly higher than control (p < 0.05)

(Using the Student’s t-test)
Table 2.6 Ornithine Decarboxylase (ODC) Activity at day 90, in the Intestine and Liver of Rats in the Initiation Study on Quercetin and Shikimate using MNNG and Lithocholate

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}\text{CO}_2$/30 minutes/mg protein)</th>
<th>Intestine</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initiator</td>
<td>Promoter</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Lithocholate$^a$</td>
<td>10±2</td>
<td>9±3</td>
</tr>
<tr>
<td>MNNG</td>
<td>Lithocholate</td>
<td>28±5*</td>
<td>9±1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Lithocholate</td>
<td>65±19*</td>
<td>29±10</td>
</tr>
<tr>
<td>Shikimate</td>
<td>Lithocholate</td>
<td>26±4*</td>
<td>6±2</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

a - control group

* - value significantly higher than control (p < 0.05)

(Using the Student's t-test)

In the promotion studies, the cyclophosphamide plus quercetin, BBN plus quercetin and MNNG plus quercetin elevated ODC levels in the tissues examined though in the last-named group, it did not apply to the intestine (Tables 2.7, 2.8 and 2.9). The BBN plus shikimate treatment elevated the levels in the liver but not in the bladder, while the MNNG plus shikimate treatment elevated the ODC level in the intestine but not in the liver (Tables 2.8 and 2.9).
Table 2.7 Ornithine Decarboxylase (ODC) Activity at day 90, in the Bladder and Liver of Rats in the Promotion Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Promoter</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>a</td>
<td>116±5</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Cyclophosphamide</td>
<td>149±7*</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Quercetin</td>
<td>1100±366*</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Shikimate</td>
<td>206±69</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - control group
* - value significantly higher than control (p < 0.05)
(Using the Student’s t-test)

Table 2.8 Ornithine Decarboxylase (ODC) Activity at day 90, in the Bladder and Liver of Rats in the Promotion Study on Quercetin and Shikimate using BBN and Saccharin

<table>
<thead>
<tr>
<th>Group</th>
<th>Promoter</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>BBN</td>
<td>a</td>
<td>446±18</td>
</tr>
<tr>
<td>BBN</td>
<td>Saccharin</td>
<td>586±183</td>
</tr>
<tr>
<td>BBN</td>
<td>Quercetin</td>
<td>7700±3100*</td>
</tr>
<tr>
<td>BBN</td>
<td>Shikimate</td>
<td>24±4*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - control group
+ - value significantly lower than control (p < 0.05)
* - value significantly higher than control (p < 0.05)
(Using the Student’s t-test)
Table 2.9 Ornithine Decarboxylase (ODC) Activity at day 90, in the Intestine and Liver of Rats in the Promotion Study on Quercetin and Shikimate using MNNG and Lithocholate

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intestine</td>
</tr>
<tr>
<td>MNNG</td>
<td>a</td>
<td></td>
<td>29±3</td>
</tr>
<tr>
<td>MNNG</td>
<td>Lithocholate</td>
<td></td>
<td>28±5</td>
</tr>
<tr>
<td>MNNG</td>
<td>Quercetin</td>
<td></td>
<td>26±11</td>
</tr>
<tr>
<td>MNNG</td>
<td>Shikimate</td>
<td></td>
<td>124±41*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - control group
* - value significantly higher than controls (p < 0.05) (Student’s t-test)

Table 2.10 Comparison of ODC values of positive control (BBN) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control</td>
<td>142±19</td>
</tr>
<tr>
<td>BBN</td>
<td>446±18*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - Distilled water administered by gavage
* - value significantly higher than control (p < 0.05) (Using the Student’s t-test)
### Table 2.11 Comparison of ODC values of positive control (Saccharin) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control$^a$</td>
<td>151±22</td>
</tr>
<tr>
<td>Saccharin</td>
<td>597±101$^*$</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

$^a$ - Rats fed normal rodent diet

$^*$ - Value significantly higher than control ($p < 0.05$)

(Using the Student's t-test)

### Table 2.12 Comparison of ODC values of positive control (Cyclophosphamide) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control$^a$</td>
<td>148±18</td>
</tr>
<tr>
<td>Cyclophosphamide$^b$</td>
<td>116±5</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

$^a$ - Distilled water administered intraperitoneally

$^b$ - Cyclophosphamide administered as initiator

$^*$ - Value significantly higher than control ($p < 0.05$)

(Using the Student's t-test)
### Table 2.13 Comparison of ODC values of positive control (Cyclophosphamide) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
<td>Liver</td>
</tr>
<tr>
<td>Control$^a$</td>
<td>148±18</td>
<td>3±1</td>
</tr>
<tr>
<td>Cyclophosphamide$^b$</td>
<td>193±7*</td>
<td>13±1*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

- $^a$ Distilled water administered intraperitoneally
- $^b$ Cyclophosphamide administered as promoter
- * Value significantly higher than control (p < 0.05)
(Using the Student's t-test)

### Table 2.14 Comparison of ODC values of positive control (MNNG) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intestine</td>
<td>Liver</td>
</tr>
<tr>
<td>Control$^a$</td>
<td>12±2</td>
<td>4±1</td>
</tr>
<tr>
<td>MNNG</td>
<td>29±3*</td>
<td>5±1</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

- $^a$ Distilled water administered by gavage
- * Value significantly higher than control (p < 0.05)
(Using the Student's t-test)
**Table 2.15** Comparison of ODC values of positive control (lithocholate) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intestine</td>
</tr>
<tr>
<td>Control$^a$</td>
<td>14+2</td>
</tr>
<tr>
<td>Lithocholate</td>
<td>10+2</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

$^a$ - Distilled water administered by gavage

No statistical differences (Using the Student's t-test)

**Table 2.16** Comparison of ODC values of dietary control with gavage control

| Group               | ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein) |   |
|---------------------|--------------------------------------------------------|
|                     | Bladder                  | Liver         |   |
| Control (diet)      | 151+22                   | 3+1           |   |
| Control (gavage)    | 142+19                   | 4+1           |   |

Values are the means ± S.E.M. of five rats.

No statistical differences (Using the Student's t-test)

**Table 2.17** Comparison of ODC values of gavage control with intraperitoneal control

| Group            | ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein) |   |
|------------------|--------------------------------------------------------|
|                  | Bladder                  | Liver         |   |
| Control (gavage) | 142+19                   | 4+1           |   |
| Control (i/p)    | 148+18                   | 3+1           |   |

Values are the means ± S.E.M. of five rats.

No statistical differences (Using the Student's t-test)
### Table 2.18 Comparison of ODC values of dietary control with intraperitoneal control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
<th>Bladder</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (diet)</td>
<td>151±22</td>
<td></td>
<td>3±1</td>
</tr>
<tr>
<td>Control (i/p)</td>
<td>148±18</td>
<td></td>
<td>3±1</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

No statistical differences (Using the Student's t-test)

### Table 2.19 Comparison of ODC values of dietary control with gavage control

<table>
<thead>
<tr>
<th>Group</th>
<th>ODC Activity (pmole $^{14}$CO$_2$/30 minutes/mg protein)</th>
<th>Intestine</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (gavage)</td>
<td>12±2</td>
<td></td>
<td>4±1</td>
</tr>
<tr>
<td>Control (diet)</td>
<td>14±2</td>
<td></td>
<td>3±1</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

No statistical differences (Using the Student's t-test)
2.11.11 Plasminogen Activator (PA)

The quercetin-treated group had an elevated plasminogen activator level in the bladder only, while the level of plasminogen activator in the shikimate-treated group was lower in the liver (Table 2.20).

Table 2.20 Plasminogen Activator (PA) Activity at day 90 in Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets in the Sub-Acute Study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bladder</th>
<th>Liver</th>
<th>Intestine</th>
<th>Spleen</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.0±8.1</td>
<td>10.2±4.2</td>
<td>20.4±9.2</td>
<td>7.0±2.6</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>50.0±4.0*</td>
<td>2.1±1.4</td>
<td>31.0±12.0</td>
<td>2.1±1.1</td>
<td>5.3±3.0</td>
</tr>
<tr>
<td>Bracken</td>
<td>12.0±2.6</td>
<td>3.0±0.9</td>
<td>10.4±3.0</td>
<td>12.5±4.0</td>
<td>6.0±2.4</td>
</tr>
<tr>
<td>Shikimate</td>
<td>26.0±15.0</td>
<td>0.8±0.5†</td>
<td>20.0±8.2</td>
<td>12.0±4.4</td>
<td>3.8±2.0</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
* - value significantly higher than control (p < 0.05)
† - value significantly lower than control (p < 0.05)
(Using the Student's t-test)
Table 2.21 Plasminogen Activator (PA) Activity at day 90, in the Bladder and Liver of Rats in the Initiation Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Promoter</th>
<th>PA Activity (IU/mg protein) x 10²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>-</td>
<td>Cyclophosphamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.8±0.6</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Cyclophosphamide</td>
<td>21.6±1.8</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Cyclophosphamide</td>
<td>N.D.&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Shikimate</td>
<td>Cyclophosphamide</td>
<td>20.3±4.2</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

<sup>a</sup> - control group

N.D. - Not Detected

<sup>*</sup> - value significantly higher than controls (p < 0.05)

<sup>+</sup> - value significantly lower than controls (p < 0.05)

(Using the Student's t-test)

In the initiation studies the quercetin plus cyclophosphamide treatment depressed plasminogen activator levels in the bladder and liver (Table 2.21), the quercetin plus saccharin treatment elevated the liver levels of plasminogen activator as did the shikimate plus saccharin treatment but in the bladder this treatment depressed plasminogen activator levels (Table 2.22). However, the quercetin plus lithocholate and shikimate plus lithocholate treatments both elevated intestinal plasminogen activator levels but while the quercetin plus lithocholate treatment also elevated the liver levels, the shikimate plus lithocholate treatment made no difference to the liver levels (Table 2.23).
Table 2.22 Plasminogen Activator (PA) Activity at day 90, in the Bladder and Liver of Rats in the Initiation Study on Quercetin and Shikimate using BBN and Saccharin

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bladder</td>
<td>Liver</td>
</tr>
<tr>
<td>-</td>
<td>Saccharin^a</td>
<td>40.5±2.6</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>BBN</td>
<td>Saccharin</td>
<td>N.D. +</td>
<td>2.7±0.1*</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Saccharin</td>
<td>62.3±27.0</td>
<td>6.8±1.7*</td>
</tr>
<tr>
<td>Shikimate</td>
<td>Saccharin</td>
<td>9.3±0.6+</td>
<td>1.0±0.1*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

a - control group

N.D. - Not Detected

* - value significantly higher than controls (p < 0.05)

+ - value significantly lower than controls (p < 0.05)

(Using the Student's t-test)

Table 2.23 Plasminogen Activator (PA) Activity at day 90, in the Intestine and Liver of Rats in the Initiation Study on Quercetin and Shikimate using MNNG and Lithocholate

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intestine</td>
<td>Liver</td>
</tr>
<tr>
<td>-</td>
<td>Lithocholate^a</td>
<td>1.1±0.1</td>
<td>4.5±0.1</td>
</tr>
<tr>
<td>MNNG</td>
<td>Lithocholate</td>
<td>31.0±21.0*</td>
<td>3.3±0.1+</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Lithocholate</td>
<td>34.8±5.4*</td>
<td>5.9±0.2*</td>
</tr>
<tr>
<td>Shikimate</td>
<td>Lithocholate</td>
<td>40.4±5.3*</td>
<td>2.3±1.7</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

a - control group

* - value significantly higher than controls (p < 0.05)

+ - value significantly lower than controls (p < 0.05) (Student's t-test)
In the promotion studies, the bladder level of plasminogen activator in the BBN plus shikimate treatment was elevated but the liver level was depressed (Table 2.24). This pattern was reversed with the cyclophosphamide plus shikimate treatment (Table 2.25). Both the MNNG plus quercetin and MNNG plus shikimate treatments depressed intestinal and liver plasminogen activator levels (Table 2.26).

**Table 2.24** Plasminogen Activator (PA) Activity in the Bladder and Liver of Rats in the Promotion Assay on Quercetin and Shikimate using BBN and Saccharin

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>PA Activity (IU/mg protein) x 10²</th>
<th>Bladder</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBN a</td>
<td>-</td>
<td>N.D.</td>
<td>7.5±0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBN</td>
<td>Saccharin</td>
<td>N.D.</td>
<td>2.7±0.1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBN</td>
<td>Quercetin</td>
<td>N.D.</td>
<td>5.7±1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBN</td>
<td>Shikimate</td>
<td>127.5±78.0*</td>
<td>2.1±1.1*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

a - control group

* - value significantly higher than controls (p < 0.05)

+ - value significantly lower than controls (p < 0.05)

(Using the Student’s t-test)
Table 2.25 Plasminogen Activator (PA) Activity at day 90, in the Bladder and Liver Rats in the Promotion Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10²</th>
<th>Bladder</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide⁵</td>
<td>17.9±0.9</td>
<td>8.4±0.5</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>21.6±1.8</td>
<td>9.3±0.8</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>N.D.⁺</td>
<td>7.5±3.2</td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0.6±0.1⁺</td>
<td>10.2±0.1⁺</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
- a - control group
* - value significantly higher than controls (p < 0.05)
+ - value significantly lower than controls (p < 0.05)
(Using the Student's t-test)

Table 2.26 Plasminogen Activator (PA) Activity at day 90, in the Intestine and Liver of Rats in the Promotion Study on Quercetin and Shikimate using MNNG and Lithocholate

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10²</th>
<th>Intestine</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNNG ³</td>
<td>19.8±0.2</td>
<td>7.1±0.2</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>31.0±21.0</td>
<td>3.3±0.1⁺</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>N.D.⁺</td>
<td>4.4±0.2⁺</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>12.5±0.17⁺</td>
<td>4.4±0.1⁺</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
- a - control group
+ - value significantly lower than controls (p < 0.05)
(Using the Student's t-test)
<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10²</th>
<th>Bladder</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control²</td>
<td>29.4±10.9</td>
<td>9.3±3.3</td>
<td></td>
</tr>
<tr>
<td>BBN</td>
<td>N.D.</td>
<td>7.5±0.1</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

² - Distilled water administered by gavage
N.D. - Not Detected
No statistical differences (Using the Student's t-test)

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10²</th>
<th>Bladder</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control²</td>
<td>28.1±8.1</td>
<td>10.2±4.2</td>
<td></td>
</tr>
<tr>
<td>Saccharin</td>
<td>40.5±2.6</td>
<td>0.3±0.1²</td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

² - Rats fed normal rodent diet
² - value significantly lower than controls (p < 0.05)
(Using the Student's t-test)
### Table 2.29 Comparison of Plasminogen Activator (PA) values of positive control (Cyclophosphamide) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control^a</td>
<td>30.0±10.4</td>
</tr>
<tr>
<td>Cyclophosphamide^b</td>
<td>17.9±0.9</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

- a - Distilled water administered intraperitoneally
- b - Cyclophosphamide administered as initiator

No statistical differences (Using the Student's t-test)

### Table 2.30 Comparison of Plasminogen Activator (PA) values of positive control (Cyclophosphamide) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control^a</td>
<td>30.0±10.4</td>
</tr>
<tr>
<td>Cyclophosphamide^b</td>
<td>18.8±0.6</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

- a - Distilled water administered intraperitoneally
- b - Cyclophosphamide administered as promoter

No statistical differences (Using the Student's t-test)
### Table 2.31 Comparison of Plasminogen Activator (PA) values of positive control (MNNG) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intestine</td>
</tr>
<tr>
<td>Control</td>
<td>21.3±9.8</td>
</tr>
<tr>
<td>MNNG</td>
<td>19.8±0.2</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - Distilled water administered by gavage
No statistical differences (Using the Student's t-test)

### Table 2.32 Comparison of Plasminogen Activator (PA) values of positive control (Lithocholate) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intestine</td>
</tr>
<tr>
<td>Control</td>
<td>20.4±9.2</td>
</tr>
<tr>
<td>Lithocholate</td>
<td>1.1±0.1⁺</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - Rats fed on normal rodent diet
⁺ - value significantly lower than control (p < 0.05)
(Using the Student's t-test)
Table 2.33 Comparison of Plasminogen Activator (PA) values of dietary control with gavage control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control (Diet)</td>
<td>28.1±8.1</td>
</tr>
<tr>
<td>Control (Gavage)</td>
<td>29.4±10.9</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
No statistical differences (Using the Student's t-test)

Table 2.34 Comparison of Plasminogen Activator (PA) values of intraperitoneal control with gavage control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control (i/p)</td>
<td>30.0±10.4</td>
</tr>
<tr>
<td>Control (Gavage)</td>
<td>29.4±10.9</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
No statistical differences (Using the Student's t-test)

Table 2.35 Comparison of Plasminogen Activator (PA) values of dietary control with intraperitoneal control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bladder</td>
</tr>
<tr>
<td>Control (Diet)</td>
<td>28.1±8.1</td>
</tr>
<tr>
<td>Control (i/p)</td>
<td>30.0±10.4</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
No statistical differences (Using the Student's t-test)
Table 2.36 Comparison of Plasminogen Activator (PA) values of dietary control with gavage control

<table>
<thead>
<tr>
<th>Group</th>
<th>PA Activity (IU/mg protein) x 10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intestine</td>
</tr>
<tr>
<td>Control (Gavage)</td>
<td>21.3±9.8</td>
</tr>
<tr>
<td>Control (Diet)</td>
<td>20.4±9.2</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
No statistical differences (Using the Student's t-test)

2.11.12 Haematological Parameters

Blood Films

The thin blood films were evaluated for both the differential absolute leucocyte counts and to gain an impression of the nature of the cell types for each experimental group. The control blood films had small clusters of platelets spread evenly throughout the film. The red cells were evaluated as being normochromic, normocytic cells with a few poikilocytes. No Howell-Jolly Bodies or other inclusions were detected, nor was Rouleaux formation evident.

The predominant leucocyte in the control group was the mature lymphocyte, identified by its large nucleus which was pushed to one side and a small cytoplasmic space. The immature lymphocyte was seen occasionally as a large cell which consisted almost entirely of nucleus. In either case the nuclear chromatin was clear and contained azurophilic granules but was more dense in the mature cells than in the immature cells.

The monocyte was seen only rarely as a large cell with an indentation on one side. The cytoplasm was vacuolated and contained some azurophilic granules.

The neutrophil had a lobed nucleus with a pale blue to colourless cytoplasm and small characteristic granules. A few young neutrophils were evident by their "doughnut" ring nucleus.
The eosinophil had a bilobed nucleus, and contained deeply acidophilic staining granules. They appeared to be larger than neutrophils.

Basophils were rare but identifiable by their lobed nucleus and large dark granules.

In the quercetin, bracken and shikimate test groups as well as the cyclophosphamide (initiator, promoter and initiator-promoter), cyclophosphamide plus quercetin, cyclophosphamide plus shikimate and shikimate plus cyclophosphamide group, the degree of polychromasia was not marked relative to the control group but in the quercetin plus cyclophosphamide group this was more marked. In all these groups except the quercetin plus cyclophosphamide group the mature erythrocyte was the main red cell while in the quercetin plus cyclophosphamide the immature erythrocyte was more common. Anisocytosis was not marked in any group but the cells were generally normochromic although in cyclophosphamide plus shikimate, shikimate plus cyclophosphamide and the cyclophosphamide (initiator-promoter) groups there were some erythrocytes with only a thin ring of haemoglobin on the periphery. There were no Heinz bodies but in the bracken, shikimate, quercetin, cyclophosphamide (initiator-promoter), cyclophosphamide plus shikimate and shikimate plus cyclophosphamide groups there were varying degrees of Rouleaux formation. In addition, platelets were few and far between in the test groups.

The predominant leucocyte was the mature lymphocyte in all the test groups. Furthermore, in the various cell types there was a predominance of mature cells. The polymorphonuclear cells had characteristically staining granules as did the monocytes.

Haemoglobin

The bracken-fed groups had a statistically significantly lower haemoglobin level than the control, but while the shikimate and quercetin treated groups were not statistically lower, they revealed a biological downward trend relative to the control (Table 2.37). When compared to the cyclophosphamide-treated group either as initiator or promoter, the cyclophosphamide plus quercetin or shikimate, and quercetin or shikimate
plus cyclophosphamide were not statistically significantly different from the cyclophosphamide control, but the cyclophosphamide controls were both lower than the control (i/p) group (Tables 2.41, 2.44, 2.49 and 2.50).

Table 2.37 Some Haematological Parameters at day 90, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets in the Sub-Acute Study

<table>
<thead>
<tr>
<th>Group</th>
<th>Haemoglobin (g %)</th>
<th>PCV (%)</th>
<th>Erythrocytes (Total) mm$^{-3}$(x10$^6$)</th>
<th>Leucocytes (Total) mm$^{-3}$</th>
<th>Platelets mm$^{-3}$(x10$^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.3±1.0</td>
<td>44.6±0.9</td>
<td>7.9±0.3</td>
<td>7524±833</td>
<td>3.9±0.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>12.9±0.8</td>
<td>38.8±1.3</td>
<td>4.7±0.9†</td>
<td>3742±613†</td>
<td>3.0±0.2†</td>
</tr>
<tr>
<td>Bracken</td>
<td>11.5±0.9†</td>
<td>42.6±0.7</td>
<td>5.1±0.1†</td>
<td>5739±368</td>
<td>3.0±0.3†</td>
</tr>
<tr>
<td>Shikimate</td>
<td>14.4±0.5</td>
<td>44.6±1.3</td>
<td>3.9±0.3†</td>
<td>2977±239†</td>
<td>3.1±0.3†</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
+ - value significantly lower than controls a and b (p < 0.05)
(Using the Student's t-test)

Table 2.38 Derived Haematological Indices at day 90, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets in the Sub-Acute Study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Cell Volume MCV (fm)</th>
<th>Mean Cell Haemoglobin MCH (pg)</th>
<th>Mean Corpuscular Haemoglobin Concentration MCHC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56.2±0.5</td>
<td>18.7±0.3</td>
<td>33.0±1.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>78.0±1.5</td>
<td>26.0±0.9</td>
<td>33.0±0.4</td>
</tr>
<tr>
<td>Bracken</td>
<td>84.3±0.6</td>
<td>23.5±0.9</td>
<td>27.0±1.5</td>
</tr>
<tr>
<td>Shikimate</td>
<td>112.5±2.5</td>
<td>35.5±0.3</td>
<td>31.0±0.3</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
### Table 2.39
Total Reticulocyte Counts at day 90, of Rats fed 1\% (w/w) Quercetin, 10\% (w/w) Bracken, 1\% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88000±88</td>
</tr>
<tr>
<td>Quercetin</td>
<td>65000±130(^+)</td>
</tr>
<tr>
<td>Bracken</td>
<td>76500±306(^+)</td>
</tr>
<tr>
<td>Shikimate</td>
<td>48000±96(^+)</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
+ = values statistically lower than control (p<0.05)
(Using the Student's t-test)

### Table 2.40
Absolute Differential Leucocyte Counts at day 90, of Rats fed 1\% (w/w) Quercetin, 10\% (w/w) Bracken, 1\% (w/w) Shikimate and Control Diets in the Sub-Acute Study

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>226±68</td>
<td>45±15</td>
<td>1053±45</td>
<td>45±15</td>
<td>6170±75</td>
</tr>
<tr>
<td>Quercetin</td>
<td>165±39</td>
<td>15±7</td>
<td>636±150(^+)</td>
<td>75±11</td>
<td>2844±187(^+)</td>
</tr>
<tr>
<td>Bracken</td>
<td>80±11</td>
<td>34±11</td>
<td>976±80</td>
<td>172±17(^*)</td>
<td>4476±57(^+)</td>
</tr>
<tr>
<td>Shikimate</td>
<td>71±12</td>
<td>6±6(^+)</td>
<td>536±30(^+)</td>
<td>48±6</td>
<td>2292±30(^+)</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
* - value significantly higher than control (p < 0.05)
+ - value significantly lower than control (p < 0.05)
(Using the Student's t-test)
Table 2.41 Some haematological parameters at day 90, of Rats in the Initiation Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>Haemoglobin (9 %)</th>
<th>PCV (%)</th>
<th>Erythrocytes (Total) mm$^{-3}$ (x10$^6$)</th>
<th>Leucocytes (Total) mm$^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>CP$^a$</td>
<td></td>
<td>11.5±0.8</td>
<td>33.2±1.2</td>
<td>5.1±0.2</td>
<td>2966±51</td>
</tr>
<tr>
<td>CP</td>
<td>CP</td>
<td></td>
<td>9.8±0.8</td>
<td>35.2±1.8</td>
<td>4.6±0.1</td>
<td>2601±121</td>
</tr>
<tr>
<td>Quercetin</td>
<td>CP</td>
<td></td>
<td>11.5±1.1</td>
<td>40.2±0.3*</td>
<td>4.7±0.3</td>
<td>2420±252</td>
</tr>
<tr>
<td>Shikimate</td>
<td>CP</td>
<td></td>
<td>13.4±1.6</td>
<td>38.0±0.3+</td>
<td>3.8±0.3+</td>
<td>3362±987</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
CP - Cyclophosphamide
$^a$ - Control group
* - value significantly higher than control (p < 0.05)
+ - value significantly lower than control (p < 0.05)
(Using the Student's t-test)

Table 2.42 Derived Haematological parameters at day 90, of Rats in the Initiation Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>Mean Cell Volume MCV (fm)</th>
<th>Mean Cell Haemoglobin MCH (pg)</th>
<th>Mean Corpuscular Haemoglobin Concentration (MCHC) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>CP</td>
<td></td>
<td>64.7±0.6</td>
<td>22.5±0.2</td>
<td>36.0±0.4</td>
</tr>
<tr>
<td>CP</td>
<td>CP</td>
<td></td>
<td>70.0±2.0</td>
<td>21.3±0.8</td>
<td>28.0±0.4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>CP</td>
<td></td>
<td>80.0±1.2</td>
<td>24.4±0.5*</td>
<td>30.0±3.6</td>
</tr>
<tr>
<td>Shikimate</td>
<td>CP</td>
<td></td>
<td>100.0±3.6</td>
<td>35.2±0.6+</td>
<td>34.0±1.8</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
CP - Cyclophosphamide
**Table 2.43** Total Platelets and Reticulocytes at day 90, of Rats in the Initiation Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Platelets (x10^5 mm^-3)</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cyclophosphamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5±0.8</td>
<td>71400±142</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>3.0±0.3</td>
<td>60000±180&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.7±0.5</td>
<td>125000±4250&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Shikimate</td>
<td>3.0±0.4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>38000±38&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

* - value significantly higher than control (p < 0.05)
+ - value significantly lower than control (p < 0.05)

(Using the Student's t-test)

**Table 2.44** Some Haematological parameters at day 90, of Rats in the Promotion Study on Quercetin and Shikimate using Cyclophosphamide

<table>
<thead>
<tr>
<th>Group</th>
<th>Haemoglobin (%)</th>
<th>PCV (%)</th>
<th>Erythrocytes (Total) mm^-3 (x10^6)</th>
<th>Leucocytes (Total) mm^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.6±0.4</td>
<td>34.6±1.2</td>
<td>5.9±0.3</td>
<td>2604±149</td>
</tr>
<tr>
<td>CP</td>
<td>9.8±0.8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>35.2±1.8</td>
<td>4.6±0.1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2601±121</td>
</tr>
<tr>
<td>CP Quercetin</td>
<td>13.8±0.4</td>
<td>39.6±0.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.9±0.5</td>
<td>2635±146</td>
</tr>
<tr>
<td>CP Shikimate</td>
<td>13.4±0.7</td>
<td>39.6±1.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.5±0.2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3822±928</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

CP - Cyclophosphamide

* - value significantly higher than control (p < 0.05)
+ - value significantly lower than control (p < 0.05)

(Using the Student's t-test)
Table 2.45 Some Derived Haematological parameters at day 90, of Rats in the Promotion Study on Quercetin and Shikimate using Cyclophosphamidem

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>Mean Cell Volume (MCV) (fm)</th>
<th>Mean Cell Haemoglobin (MCH) (pg)</th>
<th>Mean Corpuscular Haemoglobin Concentration (MCHC) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide -</td>
<td>CP</td>
<td>-</td>
<td>53.3±2.0</td>
<td>23.0±0.4</td>
<td>39.3±0.3</td>
</tr>
<tr>
<td>Cyclophosphamide CP</td>
<td>CP</td>
<td>CP</td>
<td>70.0±2.0</td>
<td>21.3±0.8</td>
<td>27.8±0.4</td>
</tr>
<tr>
<td>Cyclophosphamide CP</td>
<td>Quercetin</td>
<td>CP</td>
<td>80.0±1.5</td>
<td>28.1±0.8</td>
<td>34.8±0.6</td>
</tr>
<tr>
<td>Cyclophosphamide CP</td>
<td>Shikimate</td>
<td>CP</td>
<td>88.8±1.0</td>
<td>29.1±0.7</td>
<td>33.0±0.7</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
CP - Cyclophosphamide

Table 2.46 Total Platelets and Reticulocytes at day 90, of Rats in the Promotion Study on Quercetin and Shikimate using Cyclophosphamidem

<table>
<thead>
<tr>
<th>Group</th>
<th>Initiator</th>
<th>Promoter</th>
<th>Platelets mm⁻³ (x10⁵)</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamidea -</td>
<td>-</td>
<td>-</td>
<td>3.5±0.1</td>
<td>84000±84</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Cyclophosphamide</td>
<td>-</td>
<td>3.0±0.3⁺</td>
<td>60000±180⁺</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Quercetin</td>
<td>-</td>
<td>3.2±0.3⁺</td>
<td>80000±160⁺</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Shikimate</td>
<td>-</td>
<td>3.2±0.4⁺</td>
<td>67500±135⁺</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - Control group
⁺ - value significantly lower than control (p < 0.05)
(Using the Student’s t-test)
### TABLE 2.47 Differential Absolute Leucocyte Counts at Day 90, of Rats in the Initiation Study on Quercetin and Shikimate Using Cyclophosphamide

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Initiator</th>
<th>Promoter</th>
<th>Eosinophil</th>
<th>Basophil</th>
<th>Neutrophil</th>
<th>Monocyte</th>
<th>Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>Cyclophosphamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47 ± 15</td>
<td>17 ± 6</td>
<td>189 ± 85</td>
<td>47 ± 23</td>
<td>2669 ± 89</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>Cyclophosphamide</td>
<td>31 ± 5</td>
<td>10 ± 5</td>
<td>182 ± 79</td>
<td>52 ± 17</td>
<td>2360 ± 78*</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>Cyclophosphamide</td>
<td>29 ± 5</td>
<td>10 ± 5</td>
<td>266 ± 24</td>
<td>48 ± 8</td>
<td>2057 ± 24*</td>
</tr>
<tr>
<td></td>
<td>Shikimate</td>
<td>Cyclophosphamide</td>
<td>67 ± 11</td>
<td>20 ± 7</td>
<td>369 ± 67</td>
<td>73 ± 27</td>
<td>2824 ± 33</td>
</tr>
</tbody>
</table>

Results are the mean ± S.E.M. of five rats.

<sup>a</sup> = Control Group

+ = Values significantly lower than control, p<0.05 (Student's t-test).

### TABLE 2.48 Differential Absolute Leucocyte Counts at Day 90, of Rats in the Promotion Study on Quercetin and Shikimate Using Cyclophosphamide

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Initiator</th>
<th>Promoter</th>
<th>Eosinophil</th>
<th>Basophil</th>
<th>Neutrophil</th>
<th>Monocyte</th>
<th>Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyclophosphamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>36 ± 11</td>
<td>10 ± 5</td>
<td>218 ± 85</td>
<td>57 ± 21</td>
<td>2343 ± 80</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>Cyclophosphamide</td>
<td>31 ± 5</td>
<td>10 ± 5</td>
<td>182 ± 79</td>
<td>52 ± 17</td>
<td>2340 ± 78</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>Quercetin</td>
<td>32 ± 5</td>
<td>11 ± 6</td>
<td>169 ± 79</td>
<td>53 ± 9</td>
<td>2240 ± 26</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>Shikimate</td>
<td>122 ± 42</td>
<td>53 ± 18*</td>
<td>420 ± 88</td>
<td>84 ± 31</td>
<td>3134 ± 114*</td>
</tr>
</tbody>
</table>

Results are the mean ± S.E.M. of five rats.

<sup>a</sup> = Control Group

* = Values significantly higher than control, p<0.05 (Student's t-test)
TABLE 2.49 Comparison of Some Haematological Parameters of Positive Control (Cyclophosphamide-initiator) with Appropriate Control

<table>
<thead>
<tr>
<th>Group</th>
<th>Haemoglobin (g%)</th>
<th>PLT (x10^4)</th>
<th>Total Erythrocytes (x10^6)</th>
<th>Total Leucocytes (x10^3)</th>
<th>Total Platelets (x10^3)</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controla</td>
<td>14.9 ± 1.0</td>
<td>45.0 ± 1.0</td>
<td>7.3 ± 0.5</td>
<td>6382 ± 302</td>
<td>3.6 ± 0.3</td>
<td>95,000 ± 190</td>
</tr>
<tr>
<td>Cyclophosphamideb</td>
<td>13.6 ± 0.4</td>
<td>34.6 ± 1.2</td>
<td>5.9 ± 149</td>
<td>2604 ± 149</td>
<td>3.5 ± 0.1</td>
<td>84,000 ± 84†</td>
</tr>
</tbody>
</table>

Results are the mean ± S.E.M. of five rats

a = Distilled water administered intraperitoneally
b = Cyclophosphamide administered as initiator
† = Significantly lower than control, p<0.05 (Student’s t-test).
Table 2.50 Comparison of Derived Haematological parameters of positive control (Cyclophosphamide - initiator) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Cell Volume (MCV) (fm)</th>
<th>Mean Cell Haemoglobin (MCH) (pg)</th>
<th>Mean Corpuscular Haemoglobin Concentration (MCHC) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^a)</td>
<td>61.6±2.0</td>
<td>20.5±2.0</td>
<td>33.1±0.1</td>
</tr>
<tr>
<td>Cyclophosphamide(^b)</td>
<td>58.3±2.0</td>
<td>23.0±0.4</td>
<td>39.3±0.3</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

\(^a\) Distilled water administered intraperitoneally

\(^b\) Cyclophosphamide administered as initiator

Haematocrit

The quercetin-treated group had a statistically significantly lower PCV than the control group (Table 2.37), but the quercetin plus cyclophosphamide, shikimate plus cyclophosphamide, cyclophosphamide plus quercetin and cyclophosphamide plus shikimate groups all had statistically significantly higher PCV than the appropriate cyclophosphamide controls (Tables 2.41 and 2.44). The cyclophosphamide controls were statistically lower than the intraperitoneally treated control group (Table 2.49 and 2.50).

Total Erythrocyte Counts

The quercetin, bracken and shikimate groups (Table 2.37) as well as the cyclophosphamide (initiator-promoter), cyclophosphamide plus quercetin, cyclophosphamide plus shikimate and shikimate plus cyclophosphamide groups had lower total erythrocyte counts relative to the respective controls (Tables 2.41, 2.44, 2.49 and 2.50).
Total Leucocyte Counts

The quercetin and shikimate groups had a statistically lower leucocyte count, and the bracken group showed a biological downward trend (Table 2.37). The cyclophosphamide (initiator and promoter) groups had a statistically significant leucopenia compared to the control (i/p) group (Tables 2.49 and 2.50).

Total Platelets

The quercetin, bracken and shikimate groups had a thrombocytopenia (Table 2.37) as did cyclophosphamide (initiator-promoter), cyclophosphamide plus quercetin, and cyclophosphamide plus shikimate groups (Table 2.46). With the exception of the quercetin plus cyclophosphamide group, the cyclophosphamide plus cyclophosphamide, and shikimate plus cyclophosphamide groups had a biological downward trend in their platelet counts (Table 2.43).

Reticulocytes

With the exception of the quercetin plus cyclophosphamide group (Table 2.43), all other test groups had a statistically significantly lower reticulocyte count relative to the control groups (Tables 2.43 and 2.46).

Derived Haematological Indices

The Mean Cell Volume (MCV) calculated for the quercetin, bracken and shikimate groups (Table 2.38) as well as the cyclophosphamide (initiator-promoter), quercetin plus cyclophosphamide, shikimate plus cyclophosphamide, cyclophosphamide plus quercetin, and cyclophosphamide plus shikimate groups indicate a macrocytic anaemia (Tables 2.38, 2.42 and 2.45), and the Mean Cell Haemoglobin (MCH) correlates well with the MCV in these groups. The Mean Corpuscular Haemoglobin Concentration (MCHC) however indicates a normochromasia in each of these groups.
TABLE 2.51 Comparison of SOME Haematological Parameters of Positive Control (Cyclophosphamide-Promoter) with Appropriate Control

<table>
<thead>
<tr>
<th>Group</th>
<th>Haemoglobin (g%)</th>
<th>PCV (%)</th>
<th>Total Erythrocytes mm$^{-3}$ (x 10$^6$)</th>
<th>Total Leucocytes mm$^{-3}$</th>
<th>Total Platelets mm$^{-3}$ (x 10$^3$)</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control$^a$</td>
<td>14.9 ± 1.0</td>
<td>45.0 ± 1.0</td>
<td>7.3 ± 0.5</td>
<td>6382 ± 802</td>
<td>3.6 ± 0.3</td>
<td>95,000 ± 190</td>
</tr>
<tr>
<td>Cyclophosphamide$^b$</td>
<td>11.5 ± 0.8$^+$</td>
<td>33.2 ± 1.2$^+$</td>
<td>5.1 ± 0.2$^+$</td>
<td>2966 ± 51$^+$</td>
<td>3.5 ± 0.8</td>
<td>71,400 ± 142$^+$</td>
</tr>
</tbody>
</table>

Results are the mean ± S.E.M. of five rats

$^a$ = Distilled water administered intraperitoneally

$^b$ = Cyclophosphamide administered as promoter

$^+$ = Significantly lower than control, p<0.05 (Student's t-test)
Differential Leucocyte Counts

The quercetin, bracken and shikimate groups revealed a biological downward trend in the granulocytes and lymphocytes (Table 2.40), but in conjunction with cyclophosphamide this trend was not consistently maintained especially in the shikimate plus cyclophosphamide and cyclophosphamide plus shikimate groups (Tables 2.47 and 2.48).

Table 2.52 Comparison of Derived Haematological parameters of positive control (Cyclophosphamide - promoter) with appropriate control

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Cell Volume (MCV) fm</th>
<th>Mean Cell Haemoglobin (MCH) pg</th>
<th>Mean Corpuscular Haemoglobin Concentration (MCHC) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>61.6±2.0</td>
<td>20.5±2.0</td>
<td>33.1±0.1</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>64.7±0.6</td>
<td>22.5±0.2</td>
<td>36.0±0.4</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
a - Distilled water administered intraperitoneally
b - Cyclophosphamide administered as promoter

Table 2.53 Comparison of Absolute Differential Leucocyte Counts of positive control (Cyclophosphamide - initiator) with appropriate control

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>280±35</td>
<td>25±13</td>
<td>957±191</td>
<td>50±13</td>
<td>4977±64</td>
</tr>
<tr>
<td>CP b</td>
<td>36±11+</td>
<td>10±5</td>
<td>218±85+</td>
<td>57±21</td>
<td>2343±80+</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
CP - Cyclophosphamide
a - Distilled water administered intraperitoneally
b - Cyclophosphamide administered as initiator
+ - value significantly lower than control (p < 0.05) (Using the Student's t-test)
### Table 2.54 Comparison of Absolute Differential Leucocyte Counts of positive control (Cyclophosphamide - promoter) with appropriate control

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (^a)</td>
<td>280±35</td>
<td>25±13</td>
<td>957±191</td>
<td>50±13</td>
<td>4977±64</td>
</tr>
<tr>
<td>CP (^b)</td>
<td>47±15(^+)</td>
<td>17±6</td>
<td>189±85(^+)</td>
<td>47±23</td>
<td>2669±89(^+)</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

CP - Cyclophosphamide

\(^a\) Distilled water administered intraperitoneally

\(^b\) Cyclophosphamide administered as promoter

\(^+\) Value significantly lower than control (p < 0.05)

(Using the Student’s t-test)
<table>
<thead>
<tr>
<th>Group</th>
<th>Haemoglobin (g%)</th>
<th>PCV (%)</th>
<th>Total Erythrocytes $\text{mm}^{-3}$ (x $10^9$)</th>
<th>Total Leucocytes $\text{mm}^{-3}$</th>
<th>Total Platelets $\text{mm}^{-3}$ (x $10^9$)</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Diet)</td>
<td>15.3 ± 1.0</td>
<td>44.6 ± 0.9</td>
<td>7.9 ± 0.3</td>
<td>7524 ± 833</td>
<td>3.9 ± 0.1</td>
<td>88,000 ± 88</td>
</tr>
<tr>
<td>Control (1/9)</td>
<td>14.9 ± 1.0</td>
<td>45.0 ± 1.0</td>
<td>7.3 ± 0.3</td>
<td>6382 ± 802</td>
<td>3.6 ± 0.3</td>
<td>95,000 ± 190*</td>
</tr>
</tbody>
</table>

Results are the mean ± S.E.M. of five rats

* = Significantly higher than dietary control, $p < 0.05$ (student-t-test)
Table 2.56 Comparison of Derived Haematological parameters of Dietary Control with Intraperitoneal Control

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Cell Volume (MCV) (fm)</th>
<th>Mean Cell Haemoglobin (MCH) (pg)</th>
<th>Mean Corpuscular Haemoglobin Concentration (MCHC) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (diet)</td>
<td>56.2±0.5</td>
<td>18.7±0.3</td>
<td>33.0±1.0</td>
</tr>
<tr>
<td>Control (i/p)</td>
<td>61.6±2.0</td>
<td>20.5±2.0</td>
<td>33.1±0.1</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.

Table 2.57 Comparison of Absolute Differential Leucocyte Counts of Dietary Control with Intraperitoneal Control

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>226±68</td>
<td>45±15</td>
<td>1053±45</td>
<td>45±15</td>
<td>6170±75</td>
</tr>
<tr>
<td>(diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>280±35</td>
<td>25±13</td>
<td>957±191</td>
<td>50±13</td>
<td>4977±64+</td>
</tr>
<tr>
<td>(i/p)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of five rats.
+ - value significantly lower than control (p < 0.05)
(Using the Student’s t-test)
2.12 Discussion

2.12.1 Chronic Study

The objective of the chronic study was to determine if shikimate and/or quercetin, both compounds isolated from bracken were responsible for the carcinogenicity of bracken. Although it is the accepted strategy and method in these studies to feed the compound over the entire life span of the animal (in this case, two years), this practice has not been followed here because it is more a comparative study of the carcinogenic properties of shikimate and quercetin with those of bracken rather than an empirical determination of the independent carcinogenic properties of shikimate and quercetin. Hence the two test compounds, quercetin and shikimate, were fed only for as long as the parent composite, bracken. In addition, the expense of life-time feeding fore closed that option in this study.

The 1.0% (w/w) levels used for quercetin and shikimate are higher than the levels normally obtained in bracken. Shikimate is estimated to occur at a level of 1.44 mg/g dry weight of bracken (I.A. Evans, personal communication to Hirono et al., 1977a), equivalent to 0.14%. However, this dose level had been previously employed without any tumours developing even when fed for 142 days. The use of a ten-fold increase ought, if there is a dose-related carcinogenic effect, to produce tumours in some of the experimental rats. As it is there were no tumours in any of the shikimate-fed rats up to two years old. The amount of quercetin in bracken has been estimated at 0.57 g per kg dry weight of bracken or 0.057% (Pamukcu et al., 1980b). However, various quantities of quercetin have been used and only one report of quercetin carcinogenicity has so far been recorded (Pamukcu et al., 1980b) and the failure to reproduce it elsewhere has cast doubt on the real carcinogenic activity of quercetin.

There were however certain histopathological lesions in some of the quercetin- and shikimate-fed rats which deserve a mention with regard to the age of the rats rather than as evidence of compound related toxic effects. In this regard, the foci of mononuclear cells in the kidneys of both groups as well as in the control rats are indicative of a chronic glomerulonephritis which is commonly observed in rats of this age and has been termed "rat nephropathy". The ceroid which was deposited in the spleen
may be a product of lipid peroxidation, but may also be found along with golden brown pigment and/or lipofuscin in organs undergoing cachexia or senile atrophy.

The bracken-induced ataxia which occurred in some rats at week 4, had been observed in other studies and attributed to the actions of the enzyme thiaminase (Weswig et al., 1946; Evans and Evans, 1949). This phenomenon is further examined in Chapter Three.

The intussusception which led to the sudden death of one of the bracken-fed rats was the main factor that led to the premature termination of this aspect of the Chronic Study at 8.5 months rather than at 24 months.

Intussusceptions were found in previous studies on bracken carcinogenicity (Evans and Mason, 1965; Pamukcu et al., 1980b). Intussusceptions occur as a result of abnormal peristalsis in one part of the gut so that successive portions are moving more slowly than preceding portions and the former then telescope into the latter. Bracken-induced tumours tend to occur in those parts of the gut where the gut is more prone to stagnation (Hirono et al., 1977b). These may suggest the presence of an "antimotility agent" in the bracken or may suggest that the fibrous content of bracken induces greater than usual catharsis.

The implication of intussusception that might occur in the course of a long-term carcinogenicity test ought to be borne in mind in the adoption of toxicity testing schedules and the need for flexibility in these studies. In this study, the 10% (w/w) bracken diet is already low compared to other studies where 15-70% (w/w) bracken diets have been used (Appendix I). Another measure might be to implement additional or satellite groups to take account of such an eventuality, but the additional cost, must be borne in mind.

The result of the termination of the study at 8.5 months was that benign tumours only, were induced in the experimental rats. These may progress to malignancy if left for a longer period. However, the ability of bracken to induce tumours has been demonstrated but the ability is not attributable to either quercetin or shikimate.
2.12.2 Sub-Acute Study

The studies on ornithine decarboxylase and plasminogen activator were carried out to determine the tumour promoting activities of quercetin and shikimate. The enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) catalyses the formation of the polyamine putrescine which is the precursor of spermidine. The polyamines are found in very high concentrations in those tissues with very high rates of biosynthetic activity, rapidly growing tissues, embryonic tissues and neoplasms (Russell and Snyder, 1968). The decarboxylation of ornithine to form putrescine is a rate-limiting step in the formation of polyamines and the determination of ornithine decarboxylase has been used as a sensitive and consistent index in the determination of tumour promoting activity by various chemicals (O'Brien, 1976; Hosaka et al., 1983) because the tumour promotion phase of the two-stage carcinogenesis model is the phase of rapid cell differentiation and proliferation.

"Plasminogen activator" is a term which refers to a wide variety of proteases whose ultimate biological activity is the opening of peptide linkages in fibrin and fibrinogen and clot lysis. Plasminogen activator is found in high levels in transformed cells, but unlike ornithine decarboxylase it is not an obligatory or consistent finding in transformed cells (Pitot, 1981).

In these studies the techniques used for the determination of ornithine decarboxylase is the sensitive liquid scintillation technique based on $^{14}$C. In applying it, a coefficient of variation (reproducibility) was an average of 46%, ranging from 8-93% in comparison to the range of 16-244% obtained by Ball and Balis (1976). Other studies indicate a high degree of individual variability in the application of the method in the determination of ODC (Hosaka et al., 1983). In determining plasminogen activator, the use of a chromogenic substrate is convenient, but its reproducibility is highly dependent on the type of tissue or cell system to which it is applied. In primary cell cultures a coefficient of variation of 35% was obtained compared to 0.25% in metastasizing Lewis Lung Carcinoma Cells (Whur et al., 1980). This is in contrast to a reproducibility of 52% (range 2-149%) in this study, but these were whole organs with the greater potential for the presence of potential plasminogen activator inhibitors such as antiplasmin and α2-macroglobulin (Richards et al., 1983).
The shikimate-fed rats had a consistently elevated ODC level in all the tissues examined with the exception of the bladder where it was not a statistically significant change. This is indicative of tumour promoting ability on the part of shikimate. In conjunction with other test compounds which have been used as tumour promoters and initiators, there was an inconsistent elevation of ODC in some organs but not in others. In conjunction with cyclophosphamide and saccharin, shikimate administered as an initiator demonstrated an ability to enhance ODC levels in both the bladder and liver, and in conjunction with lithocholate in the intestine but not in the liver. While it was envisaged to evaluate shikimate as an initiator in these studies, the fact that shikimate was not administered as a single dose would invalidate that criterion for evaluation of shikimate, but this strategy was adopted as a result of the need to achieve adequate doses of shikimate before the promoter was administered. This in turn suggests that it may be more valid to discuss these results not on the basis of "initiators" and "promoters" but on the basis of "cell transformation" as an umbrella term for initiation and promotion. To further buttress this is the emergent idea that initiation and promotion may not be such clearcut phenomena as hitherto thought (Bannasch, 1984), some promoters being considered as weak carcinogens. Therefore the crucial issues would appear not to be classification of compounds but on their mechanisms of actions, interactions with various other compounds and their target organs. Another development which has a bearing on this area of work is that more recent evidence points to two classes of promoters - the first stage promoter which may alter the genetic material of initiated cells in such a way that only one application would have an obligatory effect for promotion, whereas a second stage promoter would require repeated applications for the same effect. In addition to the foregoing is the concept of co-carcinogenesis in which two carcinogens may act co-operatively to induce cancer but each on its own cannot. It has been shown that papillomas induced by the initiation-promotion model differ from those induced by the application of carcinogen alone (Reddy and Fialkow, 1983).

These various concepts may be at play individually or in concert in any process of carcinogenesis. Thus the ability of shikimate on its own to enhance ODC production in various organs (Table 2.3) is indicative of tumour promoting ability but its interactions with cyclophosphamide, saccharin and lithocholate need not necessarily refer to the same process. Pertinent to
the above is the induction of glandular gastric neoplasia and leukaemia in TFI mice by shikimate. Those results, achieved by a single intraperitoneal injection or oro-gastric gavage of shikimate show that shikimate is carcinogenic to mice (Evans and Osman, 1974) but in the light of the ODC results in rats it may also be inferred that it is probably a stage one promoter in rats but in another species (mice) acts as a carcinogen.

The rest of the results of shikimate administered as a promoter may then be viewed against the background of the foregoing. Considered as a promoter, it does not enhance ODC activity relative to the cyclophosphamide (initiator) control (Table 2.7). When considered with dibutyl nitrosamine (BBN) as an initiator however, it enhanced ODC activity in the liver but not in the bladder. While the bladder may be a target organ for BBN it is noteworthy that shikimate did not enhance ODC activity in the bladder in a statistically significant way (Table 2.3). With MNNG the level of intestinal ODC was enhanced but not the liver suggesting a target organ effect probably due in the main to MNNG.

The results of shikimate with regard to plasminogen activator levels show a lowered effect on the liver of shikimate treated rats but this may be attributed to the possible action of plasminogen activator inhibitors already mentioned above. Otherwise the plasminogen activator results in Table 2.20 were largely non-statistically significant. Whereas shikimate (as initiator) in conjunction with cyclophosphamide enhanced ODC levels in the bladder and liver, it did not achieve the same with regard to plasminogen activator, but with saccharin it enhanced liver plasminogen activator levels but not bladder levels (Table 2.22). With lithocholate it enhanced intestinal levels of plasminogen activator but not liver levels. These results may illustrate the ability of shikimate to act synergistically or additively with some of the test compounds, or it may illustrate the predilection of shikimate and the test compounds concerned for certain target organs, but it must also be seen as the difference in response to a set of test substances by two markers of tumour promotion - ornithine decarboxylase and plasminogen activator. On these results, the greater reliability of ornithine decarboxylase as a marker would tend to be confirmed. When administered as a promoter, shikimate tended to act more compatibly with cyclophosphamide than when administered as an initiator (Tables 2.21 vs 2.25) with respect to the liver. The same, however, does not hold true when MNNG and lithocholate were used (Tables 2.23 vs 2.26).
The results of ODC activity as affected by quercetin show that on its own quercetin did not enhance ODC activity in any of the organs examined (Table 2.3). However, in conjunction with cyclophosphamide it enhanced ODC activity in the bladder and the liver regardless of whether it was applied before or after cyclophosphamide. In conjunction with saccharin, however, it elevated liver but not bladder ODC and in conjunction with lithocholate it enhanced intestinal but not liver ODC activity. With dibutyl nitrosamine (BBN) it enhanced both liver and bladder ODC activity but with MNNG, liver but not intestinal ODC activity was enhanced. These results may be interpreted as reflecting the ability of quercetin to act as a tumour promoter in certain organs and in conjunction with certain compounds. This view may be further buttressed by some of the plasminogen activator data such as the enhanced activity of the bladder plasminogen activator by quercetin (Table 2.20), quercetin plus saccharin (liver) (Table 2.22), and quercetin plus lithocholate (Table 2.23). These results take on added significance in view of the carcinogenicity test results which showed that quercetin could act as a carcinogen in the rat (Pamukcu et al., 1980b). The issues raised earlier with the shikimate results and the positive mouse carcinogenicity study are raised here again. That is, the possibility that quercetin may be a stage one promoter capable under certain conditions of inducing cancer.

While the above issues remain, the results of previous studies on quercetin should be recalled as these are relevant at this stage. Other studies in various animal models using varying levels of quercetin showed that quercetin is not a carcinogen (Ambrose et al., 1952; Morino et al., 1982; Takanashi et al., 1983). It was suggested that the rapid detoxification was the reason for its non-carcinogenicity (Ueno et al., 1983). Apart from its non-carcinogenicity it lacks tumour promotion capability (Fukushima et al., 1983; Kato et al., 1983; 1984). It was postulated that this effect was mediated via inhibition of lipoxygenase, an enzyme responsible for facilitating the insertion of oxygen into free fatty acids and considered to be a pre-oxidant (Kato et al., 1983). It was suggested that lipoxygenase products are essential for tumour promotion (Fischer et al., 1982). This action of quercetin is part of its inhibitory action on several enzymes like cyclooxygenase, hyaluronidase, histidine-decarboxylase, xanthine oxidase, mitochondrial bound hexokinase, ATPase and aldolase reductase amongst others (Gschwendt et al., 1983). In addition to
these, quercetin inhibits DNA, RNA and protein synthesis (Graziani and Chayoth, 1979), but tumour promoters increase DNA and RNA synthesis (Pitot, 1981).

These reports are strongly indicative of quercetin as lacking both tumour promoter and carcinogenic potential. The results of its enhancement of ODC activity in conjunction with other test compounds already adumbrated above may therefore not be attributable to any real contributions by quercetin because when tested on its own it did not produce this enhanced effect (Table 2.3). Furthermore, its effect on the plasminogen activator level of the bladder may be seen as an isolated effect and plasminogen activator is a less reliable index of tumour promotion than ornithine decarboxylase. The weight of evidence would on balance be in favour of quercetin as lacking promoter capability, but the isolated and uncorroborated report of its carcinogenicity in rats (Pamukcu et al., 1980b) remains to be clarified.

In proceeding with further studies on the biological effects of quercetin, it may be instructive to study its interaction with shikimate, a compound along with which it is a co-constituent of bracken and which has shown promoting action in this study. It is noteworthy that quercetin is mutagenic, and it would be interesting to see how this activity is related to shikimate's promoting capability in the context of bracken carcinogenicity. In this regard the role of ptaquiloside may also be examined alongside these two isolates and candidate carcinogens.

The haematological studies were carried out to examine the roles of shikimate and quercetin in the production of the haematological syndrome in bracken toxicity. The study was a 90-day sub-acute study with the determination of various haematological indices at the end of the study. This is perhaps the most important limitation of this study in that so dynamic a process as haemopoiesis requires continuous monitoring over several time points in the course of the study. This strategy may be adopted by bleeding the experimental animals from the tail vein for example, and determining the various indices required. This approach is however not without hazard especially where very young rats are used in starting the experiment as very small volumes of blood may be all that can be safely obtained from them. The use of satellite groups reserved for this purpose is a way round the problem but is an added expense.
The overall impression of the effect of quercetin, shikimate and the parent composite, bracken, of which they are consituents is a depressant effect on haemopoiesis. This is evidenced by the fatty infiltration of the bone marrow. This was more marked in the bracken-fed rats and less so in the quercetin- and shikimate-fed rats. The resultant effect of this is the reduction in the numbers of erythrocytes, leucocytes and platelets in these three groups compared to the control (Table 2.37). Although the bracken-fed rats did not reveal a statistically significantly lower number of leucocytes than the control, the overall numbers were numerically lower indicating a biologically downward trend. The lower numbers of reticulocytes in these three test groups compared to the control (Table 2.39) would indicate a non-regenerative bone-marrow damage, but it is important to view this conclusion with caution especially as this was a single time-point observation. It is not at all evident from such an observation what the events preceeding or succeeding this particular observation are and in a dynamic process such as haemopoiesis it is important or perhaps even imperative that such a continuous picture should be taken into account in considering the effects of a particular compound in the process of haemopoiesis.

These limitations notwithstanding, the test compounds quercetin and shikimate and their parent composite, bracken, induce in male Wistar-Albino rats, a macrocytic, normochromic non-regenerative anaemia (Tables 2.37 and 2.38) with bone-marrow hypoplasia.

The erythrocyte count which gave the indication of anaemia was determined in a Coulter Counter (Model Dn), with a reproducibility of 9% which is less accurate than the estimated +2% suggested for such instruments (Mitruka and Rawnsley, 1977). The Mean Cell Volume (MCV) in each of the test groups was higher than the control, but the value of 112.5 fm for shikimate is extremely high and although the impression of a macrocytic anaemia may not be in doubt, the value may reflect errors in the estimation of the erythrocyte count or the packed cell volume or both. The reproducibility of 6% for the microhaematocrit method for determination of the packed cell volume is within the range of 2.25 - 8% estimated for this method (Mitruka and Rawnsley, 1977), and it may therefore be correct to attribute any errors of estimation to the total erythrocyte count.
The bone-marrow hypoplasia is also reflected in the accompanying agranulocytopenia which was a feature of the leucopenia of the quercetin-, bracken- and shikimate-fed rats, along with a lymphocytopenia. Although the granulocytes did not always show a statistically significant difference compared to the control (Table 2.40) the numerical values were lower and reveal a biological trend. However, the monocytes of the bracken-fed rats were statistically significantly higher than the control. This may reflect a response to the agranulocytopenia which was evident in the bracken-fed rats. In the absence of the polymorphonuclear cells, the monocytes may step in and take up the defence functions of the body. However, monocytosis is a very rare phenomenon in the rat and it may be more apparent than real representing a "sympathetic drift" rather than a genuine biological effect.

The other features of this hypoplasia are the accompanying thrombocytopenia which had been observed and reported in other cases of bracken toxicity and its nonregenerative nature. However, the method of Austen and Rhymes (1975) suffers from the fact that too few platelets are used for the estimation because of the small area of the counting chamber which is used. This may be a source of errors. The lower numbers of reticulocytes is indicative of a failure to produce enough red cells and get them into the peripheral circulation. This is however only valid in the context of the time-point (i.e. at day 90) when this observation was made. Whether this is a part of a trend can only be fully determined by having a continuous picture of the events.

When either shikimate or quercetin was used in conjunction with cyclophosphamide as an initiator or as a promoter, there was a reduction in the number of erythrocytes compared to the appropriate positive control (Tables 2.41 and 2.44), but not in the leucocytes. Although the shikimate plus cyclophosphamide group had a thrombocytopenia and a reduction in the number of reticulocytes (Table 2.43) the quercetin plus cyclophosphamide group had a higher number of platelets than the cyclophosphamide (promoter) control and a reticulocytosis (Table 2.43). Although these groups had bone marrow hypoplasia, it is not clear whether these effects can be attributed to the effect of cyclophosphamide alone or quercetin alone or shikimate alone or an effect of both chemicals. This is because when used as a promoter, cyclophosphamide on its own induced a macrocytic normochromic anaemia compared to the control group (Table 2.52). In addition there was a leucopenia. This may indicate that with hindsight the dose of
cyclophosphamide used was too high. Cyclophosphamide is one of the known causes of macrocytic anaemia (Richards et al., 1983).

The quercetin plus cyclophosphamide group had a reticulocytosis (Table 2.43) which was evident as a burst of immature erythrocytes in the thin smears and an accompanying high Mean Cell Volume (MCV) of 80.0±1.2 (Table 2.42). Such a burst of reticulocytes may occur if one or two mitotic steps are missed, and the cell is prematurely thrust into the circulation. Such reticulocytes will boost the absolute reticulocyte count but are not reflective of the true state of erythropoiesis in the bone marrow. A trigger for such a phenomenon is the rapid drop in haemoglobin levels which then results in a failure of the mechanism whereby iron and erythropoietin act as the regulators of red cell synthesis. It is therefore more informative to think in terms of the corrected reticulocyte count which takes into account the 2 - 3 day reticulocyte maturation time, rather than the absolute reticulocyte count.

When used as promoter, both quercetin and shikimate induced, in conjunction with cyclophosphamide, a macrocytic, normochromic, non-regenerative anaemia with hypoplasia, and thrombocytopenia. It would appear that the two test compounds, quercetin and shikimate are capable in their own right of inducing bone marrow damage although to a lesser degree than bracken, at day 90, in male Wistar-Albino Rats.

However certain aspects of the haematology of bracken-induced bone marrow aplasia remain to be clarified. Specifically at what stage of haemopoiesis is the aplasia induced? In order to determine this, various aspects of cell kinetics will need to be investigated using some cytochemical techniques not routinely employed in haematology but which enable the cell to be studied in detail. Some of these will provide information on DNA synthesis within the cell and will enable a more accurate classification of the cell based not only on morphological features but also on histochemical properties. In addition the bone marrow damage may be induced via immunological mechanisms. In order to study this, immunofluorescence techniques may need to be employed. These could show what sort of immunoglobulins are present on the cell surface, and how these change qualitatively and quantitatively over a period of time in the course of feeding these test compounds.
Conclusion

The chronic study was able to confirm the well established carcino-
genicity of bracken in rats even though the study was prematurely terminated
consequent upon the intussusception which occurred in one rat at 8.5 months.
The carcinogenic properties of quercetin and shikimate could not be
established at 24 months.

The sub-acute study showed that shikimate could act as a tumour
promoter due to the enhanced ornithine decarboxylase activity in the various
tissues of rats fed shikimate. Quercetin did not elevate ornithine
decarboxylase activity in the tissues of rats and its role in bracken
carcinogenicity remains unclear.

Finally, quercetin and shikimate could induce in male Wistar-Albino
Rats at day 90, a macrocytic, normochromic, non-regenerative anaemia with
thrombocytopenia and granulocytopenia.
CHAPTER THREE

INVESTIGATION OF THE BRACKEN-INDUCED

ATAxia IN Rats
Introduction

During the chronic studies described in chapter two, seven of the twenty bracken-fed rats developed an ataxia from the 26th - 28th day after initiating bracken feeding. Previous studies had identified this clinical sign in bracken-fed rats (Evans and Evans, 1949) and horses (Roberts et al., 1949; Evans et al., 1951), and it was attributed to the presence of "an enzymic antithiamine factor" which upon bracken ingestion induced avitaminosis B<sub>1</sub> and an associated ataxia. This view was confirmed by the positive response of affected animals to thiamine therapy. In addition, the presence of a thermolabile factor in an aqueous extract of bracken capable of inactivating thiamine was demonstrated (Evans et al., 1950). These observations led to the widely held view that the neurologic ataxia observed in bracken-intoxicated animals was due to the action of this enzyme known as thiaminase.

However, due cognisance may not have been taken of the presence in bracken of the cyanogenic glycoside prunasin which upon hydrolysis will yield cyanide, which is also capable of inducing neurological ataxia in man and animals (Osuntokun, 1968; 1969; 1970; Blood et al., 1979; Ballantyne, 1983). It was decided therefore to investigate the role of the cyanogenic glycoside, prunasin, in bracken-induced neurologic ataxia in rats.

The influence of nitrite, a known antidote of cyanide was also examined.

The metabolism of cyanide by the enzyme rhodanese was examined and the influence of the bracken isolates, quercetin and shikimate on the metabolism of cyanide was also examined.

It is not however being suggested that either quercetin or shikimate is responsible for the bracken-induced ataxia of monogastrics.
3.2 Anti-Thiamine Factors

3.2.1 Thiaminase I (thiamine: base, 2-methyl-4-amidopyrimidine-5methyl transferase, EC. 2.5.1.2)

Thiaminase I is an enzyme that catalyses the decomposition of thiamine by a base exchange reaction involving a nucleophilic displacement on the methylene group of the pyrimidine moiety. This reaction requires co-substrates which may be primary or secondary amines, a sulphydryl group or sulphinic acid. In addition, depending on the nature of the predominant co-substrate which participates in the reaction, the thiamine analogue formed could act as a thiamine antagonist thus exacerbating the onset of thiamine deficiency (Evans, 1976).

3.2.2. Thiaminase II (thiamine hydrolase, EC. 3.5.99.2)

Thiaminase II has a narrower distribution in nature, occurring mainly in micro-organisms and is not considered important with regard to bracken.

3.2.3. Other Anti-Thiamine Factors

Other anti-thiamine factors found in bracken are rutin, iso-quercitrin and astragalin-type flavonol glucosides (Hasegawa et al., 1956, 1957), caffeic acid (3,4-dihydroxy-cinnamic acid) (Somogyi and Beruter, 1967; Somogyi, 1971), and caffeoylshikimic acid (Fukuoka, 1982). These are thought to be active only in vitro.

These have been designated "thermostable anti-thiamine factors". Bracken dried at 100°C in dry-heat still retains anti-thiamine activity (Evans et al., 1950) and it is quite probable that these thermostable anti-thiamine factors also contribute to the anti-thiamine activity of bracken, and the bracken-induced ataxia of monogastrics. One of these thermostable anti-thiamine factors called "the SF Factor" is active in vivo (Konishi, 1984).

The finding of other anti-thiamine factors in bracken reinforces the need to determine the role of other factors in the bracken in the context of
Table 3.1

Sources of Thiaminase and its Effects on Animals

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Clinico-Pathologic Signs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyprinidae (carp viscera)</td>
<td>silver fox</td>
<td>Chastek paralysis</td>
<td>Evans et. al., (1942)</td>
</tr>
<tr>
<td>Pteridophytes - bracken fronds</td>
<td>rat</td>
<td>ataxia</td>
<td>Weswig et. al., (1946)</td>
</tr>
<tr>
<td></td>
<td>horse</td>
<td></td>
<td>Evans and Evans (1949)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Roberts et. al., (1949)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Evans et. al., (1951)</td>
</tr>
<tr>
<td>- bracken rhizomes</td>
<td>pig</td>
<td>unthriftiness</td>
<td>Harding (1972)</td>
</tr>
<tr>
<td></td>
<td>sheep</td>
<td>cerebro-cortical necrosis</td>
<td>Evans et. al., (1975)</td>
</tr>
<tr>
<td>Horsetail (Equisetum spp)</td>
<td>horse</td>
<td>ataxia</td>
<td>Forenbacher (1950)</td>
</tr>
<tr>
<td>Micro-organisms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. thiaminolyticus</td>
<td>human</td>
<td>hypovitaminosis B₁ (a form of beri-beri)</td>
<td>Matsukawa et. al., (1954)</td>
</tr>
<tr>
<td>Cl. thiaminolyticum</td>
<td>cats, rats</td>
<td>hypovitaminosis B₁</td>
<td></td>
</tr>
<tr>
<td>B. thiaminolyticus</td>
<td>hens, rabbits</td>
<td>hypovitaminosis B₁</td>
<td>Kimura (1965)</td>
</tr>
<tr>
<td>B. aneurolyticus</td>
<td>guinea-pigs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl. sporogen</td>
<td>sheep, cattle</td>
<td>cerebro-cortical necrosis</td>
<td>Shreeve and Edwin (1974)</td>
</tr>
</tbody>
</table>

(After Evans, 1976)
1. Pathway of thiaminase I action utilising HNR_1R_2, R.Sh and R.SO_2H compounds as co-substrates.

2. Pathway of thiaminase II action.

(After Evans, 1976)
bracken-induced ataxia and hence the investigation into the role of the cyanogenic glycoside, prunasin.

3.3 The Anti-Thiamine Activity of Bracken

The anti-thiamine activity of bracken is mainly attributable to thiaminase I and is manifested in rats and horses by ataxia, as inappetence, listlessness and unthriftiness in pigs and as cerebro-cortical necrosis in sheep and cattle.

The enzyme attains highest levels in the bracken rhizome in winter, drops during the spring and rises again in May reaching its peak level in the autumn. The frond however has very high activity in April dropping in May and remains low during the autumn.

The clinical signs of avitaminosis B_{1} are due to the role of thiamine in the biochemical pathways of the cell. The enzymes, pyruvate decarboxylase and α-ketoglutarate decarboxylase both require thiamine pyrophosphate (TPP) as a co-factor for their actions. The biochemical lesions of low tissue thiamine levels, high blood pyruvate, increased pyruvate kinase and enhanced thiamine pyrophosphate effect in the blood transketolase reaction are manifested clinically as ataxia, anorexia, opisthotonus and clonic spasms.

The mechanism by which the biochemical lesions are translated into clinical signs is not certain but some suggestions have been made - a failure to supply adequate energy to the cell which is starved of TPP, or a failure to produce an essential intermediate necessary for the nervous system and cardiovascular functions in thiamine deficiency. This later explanation is more plausible because of the observation that pigeon brains suspended in pyruvic acid utilised oxygen far more rapidly when thiamine was added than when it was not (Peters, 1936). This has been called the "catatorulin effect". The third explanation is that the inactivation of thiamine may result in one or more reaction sequences being inhibited leading to an accumulation of toxic factors leading to ataxia (Handler, 1958).
Although the avitaminosis $B_1$, due to the anti-thiamine factors has been reported in both ruminants and monogastrics, the clinical signs in ruminants tend to be less dramatic than in monogastrics. Whereas in monogastrics, ataxia, inappetence and listlessness are the principal signs, in ruminants, ataxia occurs only terminally. Furthermore, in order to induce avitaminosis $B_1$ in sheep very high levels of thiaminase are required as evidenced by the use of the rhizome which has 20-30 times more thiaminase than the fronds (Evans et al., 1975).

The ataxia which is observed as a terminal sign in sheep is probably a consequence of the induced cerebro-cortical necrosis. The pathogenesis of avitaminosis $B_1$ in ruminants is chronic because of the ability of the rumen microflora to generate thiamine. Consequently, whereas thiamine levels in intoxicated monogastrics may drop rapidly, the levels in ruminants drop less rapidly.

3.4 The Cyanogenic Glycoside Content of Bracken

Following the observation of Greshoff (1908) that the fronds of ferns were cyanophoric, the cyanogenic glycoside, prunasin, was isolated from bracken (Kofod and Eyjolfsson, 1966). It was estimated that in some cases the level of hydrocyanic acid was as high as 50mg per 100g dried bracken (Moon and Rafaat, 1951b). It was suggested that its role might be to protect bracken from predators (Cooper-Driver and Swain, 1976).

Cyanogenic glycosides yield cyanide upon hydrolysis.

Figure 3.2 The Hydrolysis of Cyanogenic Glycosides
3.5 The Toxicology of Cyanide

Cyanide toxicity in farm animals normally occurs as a result of the ingestion of plants containing cyanogenic glycosides, usually at times of drought when normal forage is scarce. Such plants include sorghums (Johnson grass, Sudan grass and milo), arrow grass (*Triglochin* spp.), elderberry (*Sambucus* spp.), wild cherry (*Prunus* spp.) and the pits of apple, peach and apricot (Oehme, 1977).

In humans, cyanide toxicity results from dietary sources such as cassava (*Manihot utilissima*) (Osuntokun, 1968), cigarette smoking (Wilson, 1965a) or therapeutical preparations such as nitroprusside, a potent hypotensive, and succinonitrile, an antidepressant.

Other sources of exposure are insecticides based on organic thiocyanates, industrial processes for the manufacture of steel, paint and aluminium, as well as coal gasification and electroplating (Solomonson, 1981; Way, 1981).

3.5.1 Cyanide Toxicity in Farm Animals

Acute cyanide intoxication causes tissue asphyxia, a histotoxic anoxia, dyspnoea, cerebral anoxia, tremors and convulsions, a course of events lasting 1-2 hours.

Affected sheep and cattle moan, are anxious, restless and terminally, develop chronic convulsions. The mucosa is bright red, the pupils dilate and there is nystagmus.

In sub-acute cases, sheep may develop goitre (Blood et al., 1979) but chronic cyanide toxicity has not been reported in these species (Hill, 1973).

Pigs fed a cassava diet rich in cyanide had a lower rate of weight gain than the controls (Peixoto, 1965) due perhaps to the competition by cyanide for sulphur-containing amino acids with the body tissues (Tewe, 1985). A previous report however reported no ill-effects from feeding pigs on a cyanide-containing diet (Alba, 1937).
Young chicks show decreased growth rate when fed a 10-15% cassava meal but adult hens show no ill effects and continue to lay eggs normally (Vogt and Penner, 1963; Jalaludin and Yin, 1972).

3.5.2 Cyanide Toxicity in Experimental Animals

The clinical signs of cyanide toxicity in dogs and rats are excitability, ataxia, capillary congestion, and cyanosis. Histologically there is demyelination of the oligodendroglial cells (Rose et al., 1954; Ibrahim et al., 1963; Smith et al., 1963; Osuntokun, 1970). Goitre has also been reported in rats (Ekpechi et al., 1966).

3.5.3 Cyanide Toxicity in Humans

Acute toxicity in humans is characterised by anoxia, convulsions and if untreated, death rapidly supervenes.

Chronic toxicity is the more common form of cyanide toxicity caused usually through the repeated consumption of cyanide-rich foodstuffs like cassava (Manihot utilissima), or inhalation of cigarette smoke or cyanide-containing fumes from industrial processes.

The clinico-pathologic signs are neuropathologic, goitre and osteopathologic. Patients may exhibit one or more of these symptoms.

The neuropathologic symptoms include ataxia, bilateral optic atrophy, bilateral nerve deafness, posterior column myelopathy, muscular wasting and weakness. This syndrome was described as Tropical Ataxia Neuropathy (Osuntokun, 1968).

In some parts of eastern Nigeria where cassava is the staple diet a goitre incidence of 41.0% was related to the small quantities of cyanide ingested in their diet over long periods (Ekpechi et al., 1966). The goitrogenic potential of cassava has also been experimentally demonstrated in rats (Ekpechi, 1973).
The osteopathologic symptoms of chronic cyanide toxicity in humans have been reported as kyphosis, kyphoscoliosis, osteoarthritis and osteoporosis. The mechanism of this syndrome was thought to be due to cyanide inhibiting the enzyme ascorbic acid oxidase, an enzyme important in bone metabolism (Wilson, 1965a).

3.6 The Detoxification of Cyanide

Cyanide may be detoxified in the thyroid, through a reaction with cystine and/or through reaction with vitamin B_{12}, but the most important mechanism accounting for 80% of cyanide detoxification is by conversion to thiocyanate, a reaction which is catalysed by the enzyme rhodanese (thiosulphate sulphur transferase) which is widely distributed in the mammalian body but has its highest concentration in the liver. Its optimum pH is 7.4 and the reaction is:

\[ \text{HCN} + \text{Na}_2\text{S}_2\text{O}_3 \xrightarrow{\text{RHODANASE}} \text{HSCN} + \text{Na}_2\text{SO}_3 \]

(Himwich and Saunders, 1948; Cosby and Sumner, 1945; Wood and Cooley, 1956; Osuntokun, 1970).

3.7 The Treatment of Cyanide Toxicity

The first line of approach in the treatment of poisoning by cyanide is the maintenance of vital functions such as respiratory and cardiovascular functions in so that the body can detoxify and excrete cyanide through the normal processes. Often however, supportive therapy is not enough and specific antidotes have to be used.

Various cobalt-containing compounds - cobalt nitrate, hydroxy-cobalamin, cobalt histidine, cobalt chloride and dicobalt EDTA were originally used for treating cyanide poisoning, based on the ability of cobalt to form stable complexes with cyanide. The toxicity of cobalt has however rendered their use obsolete in favour of methaemoglobin forming substances. Methaemoglobin reacts with cyanide to form cyanmethaemoglobin, in which form cyanide is rendered inert. Nitrite has also been used in this respect as a methaemoglobin-forming substance.
Figure 3.3 The Detoxification of Cyanide by Hydroxocobalamin

Cyanide $\rightarrow$ Vitamin $B_{12}$

+ Vitamin $B_{12}$ (hydroxocobalamin) $\rightarrow$ Light

Figure 3.4 The Detoxification of Cyanide by Cystine

\[
\text{Cyanide} + \text{CN}^- \rightarrow \text{NCS}^- + \text{CH}_2\text{CH}-\text{COOH} \quad \text{NH}_2
\]

\[
\text{Cystine} + \text{Cyanide} \rightarrow \text{β-Thiocyanoalanine}
\]

\[
\text{NCS}^- + \text{CH}_2\text{CH}-\text{COOH} \quad \text{NH}_2
\]

\[
\text{Cysteine} + \text{Cyanide} \rightarrow \text{2-Iminothiazolidine-4-carboxylic acid}
\]

(Broken line refers to possible (theoretical) pathway due to the decomposition of β-thiocyanoalanine to release thiocyanate).

(Oke, 1973)
3.7.1 The Treatment of Cyanide Poisoning in Humans

First-aid treatment of affected subjects is the inhalation of one ampoule of amyl nitrite every five minutes. Hospital therapy usually consists of intravenous administration of 10 ml, 3% sodium nitrite, followed by slow intravenous administration of 50% sodium thiosulphate (25 ml). In severe, life threatening situations, 600 mg of dicobalt EDTA may be given over one minute followed by another 300 mg if there is no immediate recovery (Vale and Meredith, 1983; Volans, 1983). The use of oxygen inhalation in conjunction with the nitrite-thiosulphate therapy has been recommended to alleviate the toxicity of nitrite (Way, 1981).

3.7.2 The Treatment of Cyanide Poisoning in Domestic Animals

Cyanide toxicity in the dog has been treated by intravenous administration of 25 mg/kg body weight of sodium nitrite and 2.5 mg/kg sodium thiosulphate (Rose et al., 1954).

In cattle, 3 g of sodium nitrite plus 15 g of sodium thiosulphate in 200 ml of water administered intravenously is the preferred therapeutic measure.

In sheep, a combination of 1 g sodium nitrite, plus 2.5 g sodium thiosulphate in 50 ml of water administered intravenously is the preferred therapy.

The regime adopted for horses is similar to that for cattle (Brander and Pugh, 1971; Blood et al., 1979). In all cases it may be necessary to repeat the therapy if symptoms recur.

3.8 Strategy of the Study

The levels of cyanide and its detoxification product thiocyanate as well as the enzyme rhodanese responsible for the detoxification and the levels of the antidote of cyanide, nitrite were determined in rats fed a 10% bracken diet for 28 days. This was the length of time before ataxia was observed in the chronic study (2.11.3).
The haematology of the rats was evaluated to provide information which could complement the data obtained in the 90-day study (2.11.14).

A comparative approach was adopted by feeding two groups of rats with the bracken isolates, shikimate and quercetin and assaying for the biochemical and haematological parameters described for the bracken-fed rats.

Upon termination of the experiment, full necropsies were carried out on all rats to assess any relevant pathologic and histopathologic lesions.

3.9 Materials and Methods

3.9.1 Animals

Forty male Wistar-albino (W/A) rats (University of Surrey, Animal Unit) were assigned to four groups, so that each group had equivalent mean weights to each other (60.0 ± 5.0 gm). The rats were housed five per cage as previously described (2.10.1), and fed a 10% (w/w) bracken diet, a 1% (w/w) shikimate diet, a 1% (w/w) quercetin diet and a control diet. They were weighed weekly and observed for clinical appearance.

3.9.2 Feedstuffs and Diets

The control, bracken, quercetin and shikimate diets were made up as previously described (2.10.2).

3.9.3 Sample Collection

Urine was collected by putting the rats into metabole cages and collecting urine over a 24 hour period. This was filtered and stored at -12 °C and analysed within three days.

The rats were ether anaesthetised and terminally bled via the posterior vena cava. Blood was collected in EDTA-containing tubes for haematology and in plain glass tubes for serum, which was also stored as the urine samples.
Each rat was autopsied, tissues for histopathology were fixed in 10% buffered neutral formalin, and tissues for rhodanese assay (liver and intestine) were washed in physiological saline and collected in beakers in an ice box for the rhodanese assay.

3.9.4 Chemicals and Reagents

All chemicals and reagents were of analytical reagent grade except where otherwise specified.

3.9.5 Determination of Thiocyanate

A modification of the method of Powell (1945) was used for determining serum and urinary thiocyanate.

Potassium thiocyanate (Sigma Chemical Company, Poole, Dorset, England) was made up in distilled water as a 2 g/L solution and titrated against 20 ml of 2.924 g/L silver nitrate solution (BDH Chemicals Ltd., Poole, Dorset, England) plus 5.0 ml, 12 M nitric acid (May and Baker Ltd., Manchester, England), using 1.0 ml of saturated ferric ammonium sulphate solution (BDH Chemicals Ltd., Poole, Dorset, England) as indicator. Serial dilutions of this standardised stock solution were used for the preparation of the standard curve by mixing 1.0 ml solutions of the potassium thiocyanate with 8.0 ml distilled water and 1.0 ml of ferric nitrate (BDH Chemicals Ltd., Poole, Dorset, England) made up of 50 g ferric nitrate in 500 ml of water plus 25 ml, 12 M nitric acid and made up to 1 L with distilled water. The blank solution was 25 ml, 12 M nitric acid made up to 1 L with distilled water. Absorbance was determined in a Cecil CE 292 UV digital spectrophotometer (Cecil Instruments Ltd., Cambridge, England) at 540 nm.

The assay was linear over the range 1.0 ug/ml to 1.0 mg/ml and the standard curve was the computer-generated best-fit line obtained after linear regression analysis using a statistical package based on the University of Surrey, Prime Computer System.
For the test serum and test urine, 0.5 ml of the serum or urine was added, in duplicate, to 4.0 ml of distilled water. These were regarded as the tests, while another set, in duplicate, were similarly set up and regarded as blanks. To the tests was added 0.5 ml ferric nitrate and to the blanks, 0.5 ml of the blank solution. The absorbance was determined at 540 nm in a Cecil CE 292 UV digital spectrophotometer. The absorbance of the blank was subtracted from the absorbance of the test and the actual concentration of thiocyanate read from the calibration curve. Thiocyanate was expressed as ug per ml serum or ug per mg creatinine for urine samples.

3.9.6 Determination of Cyanide

A modification of the method of Hanker et al., (1958) was used for the determination of cyanide in serum and urine. A potassium cyanide solution (Sigma Chemical Company Lt., Poole, Dorset, England) was standardised by argentometric titration against silver nitrate as previously described (3.9.5).

The standard curve was prepared by adding to 1.0 ml of 1 M sodium hydroxide in succession, 1.0 ul potassium cyanide, 1.0 ml glycine buffer made up of 77.4 g glycine and 58.6 g sodium chloride (BDH Chemicals Ltd., Poole, Dorset, England) in 1 L of distilled water, 1.0 ml, 0.01 g/L palladium chelate solution, and 1.0 ml of 1 g/L magnesium chloride solution (BDH Chemicals Ltd., Poole, Dorset, England). After eight minutes, the fluorescence was determined in a Perkin-Elmer Fluorescence Spectrophotometer model LS-5 (Perkin-Elmer Ltd., Bucks., England) with the excitation wavelength at 358 nm and the emission wavelength at 496 nm. The assay was linear over the range 0.087 ug/ml to 3.50 ug/ml. The standard curve was the computer-generated best-fit line obtained after linear regression analysis on a statistical package based on the University of Surrey, Prime Computer System. Recovery studies were carried out by adding varying concentrations of potassium cyanide to the serum samples and treating as above. Recovery ranged from 79 to 151% and the values determined from the calibration graph were corrected accordingly.

For the determination of cyanide in urine and serum, 1.0 ul aliquots were used. In the blank, distilled water was substituted for urine or serum.
The palladium chelate was prepared by adding 0.02 M 8-hydroxy-5-quinoline sulphonic acid (Sigma Chemical Company Ltd., Poole, Dorset, England) to a 0.01 M solution of palladium chloride in 300 ml of 5% (v/v) sulphonic acid (May and Baker Ltd., Manchester, England). The solution was heated to boiling and cooled to room temperature. Saturated potassium carbonate (BDH Chemicals Ltd., Poole, Dorset, England) was added until the evolution of carbon dioxide ceased. The chelate was washed repeatedly over a filter with 10% carbonate, water, ethanol and ether in succession and finally air dried. The resulting yellow powder is the chelate and a 1.0% solution in distilled water was used in the assay for cyanide.

The method is based on the demasking of 8-hydroxy-5-quinoline sulphonic acid by cyanide from the non-fluorescent potassium bis (5-sulphoxino) palladium (II) which is the palladium chelate. The liberated 8-hydroxy-5-quinoline sulphonic acid co-ordinates with magnesium to form a fluorescent chelate whose fluorescence is a measure of the cyanide present.

Figure 3.5 Principles of the Determination of Cyanide Concentration by the Demasking of Palladium Chelates

\[
\begin{align*}
2 \text{SO}_3\text{K} &+ 4\text{CN}^- & \rightarrow & 2 \text{SO}_3\text{K} &+ \text{Pd(CN)}_4^{2-} \\
\text{O} & \downarrow \text{Pd}/2 & & \downarrow \text{Pd}/2 & \\
\text{Non-fluorescent} & & & & \text{Fluorescent}
\end{align*}
\]
3.9.7 Determination of Rhodanese Activity

The method of Wilson (1965b) was used with some modifications.

The liver and intestine were separately homogenized in ice-cold acetone using 10 ml acetone per mg tissue in a Potter-Elvehjem glass teflon homogenizer (three return strokes). The homogenate was left at 0°C for 30 minutes and centrifuged at 3,000 g for 5 minutes using a bench-top centrifuge.

The supernatant was decanted, the centrifugate resuspended in acetone and centrifuged again at 3,000 g for 5 minutes. The supernatant was decanted and the centrifugate dried in a dessicator over anhydrous calcium chloride and a shallow beaker of sulphuric acid for 24 hours. The acetone dried powder was ground in a mortar and made up as a 10 mg/ml suspension in 0.0125 M sodium thiosulphate (BDH Chemicals Ltd., Poole, Dorset, England). It was thoroughly mixed and left for 15 minutes at 0°C and centrifuged at 0°C for 30 minutes at 12,000 g in a Beckmann JZ-21 Centrifuge (Beckmann-RIIC Ltd., Bucks., England). The clear supernatant was decanted, its protein content determined (2.10.7), and the rhodanese activity determined by adding 250 ul of the to a mixture of 5.75 ml tris HCl and 5.0 ml of 0.125 M sodium thiosulphate and thoroughly stirred. After 30 seconds, 1.25 ml of 1.0 M potassium cyanide as substrate was added and 0.2 ml aliquots withdrawn at 30 seconds, 1.0 minute, and every 1.0 minute thereafter for 10 minutes and added to 0.2 ml, 40% formaldehyde (BDH Chemicals Ltd., Poole, Dorset, England) which was used to stop the reaction. 1.0 ml ferric nitrate colorimetric reagent made up of 200 g ferric nitrate added to 200 ml 65% nitric acid and made up to 1L with distilled water, was added and the absorbance determined at 460 nm in a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England).

The blank was the reaction mixture at time zero, the 0.2 ml, 40% formaldehyde being used to stop the reaction. The readings were obtained against distilled water which was used to 'zero' the spectrophotometer.

The standard curve was prepared using argentometrically standardised potassium thiocyanate. The assay was linear over the range 1 ug/ml to 150 ug/ml potassium thiocyanate and the standard curve was the computer
generated best fit line obtained after linear regression analysis in a statistical package on the University of Surrey Prime Computer System.

Rhodanese activity was determined from the standard curve and expressed as umole thiocyanate per minute per mg protein. These determinations were carried out at 20°C rather than 37°C used by Wilson (1965b).

### 3.9.8 Determination of Nitrite

Urinary nitrite was determined by a modification of the of Montgomery and Dymock (1961) which was more sensitive than the Greiss-Ilosvay method.

The standard curve was prepared by adding varying concentrations of sodium nitrite (BDH Chemicals Ltd., Poole, Dorset, England) as 20 ul to 2.5 ml sulphanilic acid solution made up of 27.2 g potassium hydrogen sulphate (Hopkin and Williams Ltd., Essex, England) and 3.46 g sulphanilic acid (BDH Chemicals Ltd., Poole, England) made up to 1 L with distilled water. After 10 minutes, 2.5 ml of 0.04% (w/v) NEDH added and after 20 minutes, the absorbance was determined at 550 nm in a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England).

The assay was linear over the range 0.3 ug/ml to 20 ug/ml and the standard curve was the computer generated best fit line obtained after linear regression analysis on a statistical package in the University of Surrey Prime Computer System. For the determination of urinary nitrite, 20 ul urine aliquots were treated as described above. Urinary nitrite was determined from the standard curve and expressed as ug per mg creatinine.

This method is based on the principle of diazotization of nitrite by sulphanilic acid and the coupling of the diazonium salt to NEDH to produce the pink colour, the intensity of which is a measure of the nitrite in the sample.

The Montgomery and Dymock (1961) method employs potassium hydrogen sulphate as the buffering agent rather than acetic acid which is used in the Greiss-Ilosvay method. The buffering capacity of potassium hydrogen sulphate is better, the intensity of the colour developed is 13% greater than in the Greiss-Ilosvay method and the blank values in the Montgomery and
Dymock method are less than half those obtained by the Greiss-Ilosvay method. As a result, only small quantities of urine were required for the determination of nitrite, 20 μl aliquots being used in this assay.

3.9.9 Determination of Urinary Creatinine

A kit assay method was used for the determination of urinary creatinine (Sigma Chemical Company Ltd., Poole, Dorset, England).

Five sets of tubes were set up in duplicate containing 0, 0.05, 0.10, 0.15 and 0.20 ml of 15 mg/100 ml creatinine standard solution. To the tubes were added 0.30, 0.25, 0.20, 0.15 and 0.10 ml distilled water. To each tube was added 3.0 ml alkaline picrate and after 5 minutes the absorbance at 500 nm was determined in a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England) using the tube containing no creatinine as the reference.

The standard curve was the computer-generated best fit line obtained after linear regression analysis using a statistical package on the University of Surrey Prime Computer System. The assay was between 0.25 and 10 μg/ml creatinine.

For the determination of creatinine in the urine samples, four tubes were set up for each sample, a blank, standard and two tests. To the blank was added 0.3 ml of water, to the standard 0.3 ml of a 3 mg/100 ml creatinine solution, and to the tests 0.3 ml of the urine sample. To all tubes was added 3.0 ml of alkaline picrate solution and after 10 minutes the initial absorbance of tests and standard were determined at 500 nm against the blank in a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England).

To each tube was added 0.1 ml acid reagent and after thorough mixing and leaving for 5 minutes, the final absorbance was determined at 500 nm.

Creatinine concentration was calculated as follows:

\[
\text{Creatinine concentration (μg/ml)} = \frac{\text{Initial absorbance of test} - \text{final absorbance of test} \times 3}{\text{Initial absorbance of standard} - \text{final absorbance of standard}}
\]
3.9.10 Haematological Evaluations

The evaluations for haemoglobin, packed cell volume, total erythrocyte and leucocyte counts, total platelet counts, total reticulocyte counts, the differential leucocyte counts as well as the derived indices (MCV, MCH and MCHC) were carried out as previously described (2.10.9 - 2.10.15).

Heinz body examinations were undertaken using the same slides as for the reticulocyte counts.

Determination of methaemoglobin was done by lysing 0.2 ml of blood in a solution of 4.0 ml, 0.1 M phosphate buffer, pH 6.8 and 6.0 Nonidet P40, 10 ml/L, as detergent (Sigma Chemical Company Ltd., Poole, Dorset, England). The lysate was divided into two portions labelled a and b. The absorbance of a was measured in a Cecil CE 292 digital UV spectrophotometer at 630 nm before and after addition of one drop of 50 g/L potassium cyanide solution. One drop of potassium ferricyanide was added to b and the absorbance determined at 630 nm, after 5 minutes. One drop of potassium cyanide, 50 g/L, was added to b and the absorbance determined at 630 nm. The blank for all determinations consisted of buffer and detergent in the same proportion as for the tests. The result was calculated as follows:

\[
\text{Methaemoglobin} = \frac{\text{Initial absorbance of } a - \text{Second absorbance of } a}{\text{Initial absorbance of } b - \text{Second absorbance of } b} \times 100
\]

The value was multiplied by 100 to give the percentage value.

Although it is advisable to carry out methaemoglobin analysis within 1 hour of collecting blood, the lysate, once prepared, could be stored for up to 24 hours at 2-4°C.

Since the samples had to be transported to the laboratory from the experimental unit the lysate was prepared immediately and used for all analyses.

3.9.11 Histopathological Evaluations

These were done as previously described (2.10.17).
3.10 Results

The results are presented as the mean values obtained from ten rats for each dietary group ± standard error of the mean after statistical analysis using the Student’s t-test.

3.10.1 Weight Changes

The bracken-fed rats had a lower rate of weight gain in comparison with the control, shikimate- and quercetin-fed rats (p < 0.001) (Figure 3.4).

3.10.2 Clinical Observations

There were no signs of illness in the control, quercetin- and shikimate-fed rats, but the bracken-fed rats had piloerection. Ataxia was not observed in any of the animals.

3.10.3 Gross Pathology

The main gross pathological finding was the lack of the subcutaneous fat in the bracken-treated rats. The other groups were generally in good condition at necropsy.

Two of the bracken-fed rats had urinary calculi in their bladders.

3.10.4 Histopathology

The liver of the bracken-fed rats had occasional foci of areas of peri-portal necrosis with mononuclear cell infiltration although there were occasional polymorphs. In these areas, the liver cell architecture was completely disorganised. The liver of the control, quercetin- and shikimate-fed rats were normal.

The lungs of the rats in all the groups had eosinophilic-staining exudate in the bronchioles accompanied in some cases by detachment of the
Weights are the mean of ten rats in each group.

(4) The bracken-fed rats were statistically significantly different from the control group, \( p < 0.001 \), throughout the study period.
cuboidal epithelial lining of the bronchioles. The bracken, shikimate and control groups had an accompanying pneumonitis and alveolitis, while in the bracken group there were areas of red hepatisation with eosinophil infiltration.

The bone marrow of the various groups was filled with red marrow, with little or no fat spaces. The predominant cell type was the megakaryocyte and in the bracken-fed rats they were especially numerous. Occasional multinucleated "giant" cells were also observed especially in the bracken-fed rats.

In the spleen, the red pulp was consistently diminished only in the bracken-treated group, while in the quercetin and shikimate-treated group there was a variation with some revealing diminished red pulp and others showing plentiful red pulp. In the quercetin-, shikimate- and bracken-treated groups there was extra-corpuscular lymphoid hyperplasia with encroachment into the littoral macrophage zone, occasional necrobiotic cells and generally increased numbers of megakaryocytes especially in the shikimate- and bracken-treated groups. Extramedullary haemopoiesis was judged to be increased only in the bracken-treated group. The capsule, trabeculae and germinal centres were normal and the splenic nodules were absent. Other lesions include the increased numbers of the mitotic figures of the quercetin group and eosinophil infiltration in the bracken group.

In the kidneys there was a wide measure of inter-individual variation within groups. While some revealed no histopathological lesions, others revealed lesions ranging from hydropic change in the proximal tubules to nephro-calcinosis in the collecting ducts, along the cortico-medullary junction. Other lesions include the occasional dilated proximal tubule, occasional focal tubular nephritis, and in the bracken-treated group, slight thickening of the parietal surface of the Bowman's Capsule affecting some glomeruli. These lesions were common to all the various groups.

3.10.5 Cyanide Levels

The serum cyanide level of the quercetin-fed rats was too low to be detectable as was the urinary level.
The bracken-fed rats had significantly higher levels of cyanide in the serum than the controls, but the urinary levels were undetectable.

The shikimate-fed rats had undetectable urinary levels of cyanide but the serum level of cyanide did not differ significantly from the control level.

3.10.6 Rhodanese Activity

The liver rhodanese activity of the quercetin-fed rats was significantly higher than the control, the bracken-fed rats had significantly lower rhodanese activity in the liver than the control, while the shikimate-fed rats did not differ significantly from the control.

The intestinal rhodanese activity was undetectable in all the groups.

Table 3.2 Cyanide, Rhodanese and Thiocyanate levels after 28 days, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Cyanide (ug/ml)</th>
<th>Liver Rhodanese (umole thiocyanate/min/mg protein)</th>
<th>Serum Thiocyanate (ug/ml)</th>
<th>Urinary Thiocyanate (ug/mg creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.125+0.003</td>
<td>0.33±0.01</td>
<td>4.2±2.0</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>N.D. +</td>
<td>0.88±0.08*</td>
<td>9.0±1.4</td>
<td>10.8±1.1*</td>
</tr>
<tr>
<td>Bracken</td>
<td>0.257±0.006*</td>
<td>N.D.</td>
<td>6.3±2.9</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>Shikimate</td>
<td>0.097±0.002</td>
<td>0.44±0.08</td>
<td>3.3±1.0</td>
<td>4.7±0.1*</td>
</tr>
<tr>
<td>Coeff. of Variation(V)</td>
<td>7.1%</td>
<td>26.6%</td>
<td>110.2%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.087</td>
<td>0.21</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
N.D. = Not detected
* - value significantly higher than controls (p < 0.05)
+ - value significantly lower than controls (p < 0.05) (Student’s t-test)
3.10.7 Thiocyanate Levels

The serum thiocyanate levels were not significantly different from each other, but the urinary thiocyanate levels of the quercetin- and shikimate-fed rats were significantly higher than the control level. The urinary thiocyanate level of the bracken-fed rats was not significantly different from the control.

3.10.8 Urinary Nitrite Levels

The urinary nitrite levels of the quercetin- and bracken-fed rats were higher than the control level but in the shikimate-fed rats it was undetectable.

Table 3.3 Urinary Nitrite Levels after 28 days, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urinary Nitrite (ug/mg creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.0±0.2*</td>
</tr>
<tr>
<td>Bracken</td>
<td>13.5±0.3*</td>
</tr>
<tr>
<td>Shikimate</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Coefficient of variation (V) 1.1%
Sensitivity 0.3 ug/ml

Values are the means ± S.E.M. of ten rats.
N.D. = Not detected
* = values statistically higher than control (p<0.05)
(Using the Student's t-test)
Table 3.4 Urinary Creatinine of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets for 28 days.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urinary Creatinine (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.51±0.04</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.82±0.02*</td>
</tr>
<tr>
<td>Bracken</td>
<td>1.21±0.01</td>
</tr>
<tr>
<td>Shikimate</td>
<td>2.03±0.03*</td>
</tr>
</tbody>
</table>

Coefficient of variation (V) 4.7%
Sensitivity 0.25 ug/ml

Values are the means ± S.E.M. of ten rats.
* - value significantly higher than control (p < 0.05) (Using the Student’s t-test)

3.10.9 Haematological Parameters

There were no Heinz bodies in any of the experimental groups.

The attempt at methaemoglobin analysis yielded unreliable results and these have been omitted.
Table 3.5 Some Haematological parameters after 28 days, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets

<table>
<thead>
<tr>
<th>Groups</th>
<th>Haemoglobin (g %)</th>
<th>PCV (%)</th>
<th>Erythrocytes/mm$^3$ (x 10$^6$)</th>
<th>Leucocytes/mm$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control$^a$</td>
<td>15.0±1.0</td>
<td>43.0±0.5</td>
<td>5.3±0.0</td>
<td>15000±271</td>
</tr>
<tr>
<td>Control$^b$</td>
<td>14.6±0.0</td>
<td>47.4±0.1</td>
<td>8.9±0.1</td>
<td>9920±759</td>
</tr>
<tr>
<td>Quercetin</td>
<td>11.5±0.2$^+$</td>
<td>34.0±0.7$^+$</td>
<td>4.1±0.0$^+$</td>
<td>6701±242$^+$</td>
</tr>
<tr>
<td>Bracken</td>
<td>14.0±0.1</td>
<td>43.0±0.4</td>
<td>5.5±0.0</td>
<td>6215±187$^+$</td>
</tr>
<tr>
<td>Shikimate</td>
<td>12.0±0.3$^+$</td>
<td>44.0±0.7</td>
<td>6.0±0.0</td>
<td>14000±347</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.

a - Experimental control from 28 day study
b - Historical control from Mitruka and Rawnsley (1977).

+ - values significantly lower than controls a and b (p < 0.05)
(Using the Student's t-test)

Table 3.6 Platelet and Reticulocyte Counts after 28 days, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets

<table>
<thead>
<tr>
<th>Groups</th>
<th>Platelets/mm$^3$ (x 10$^5$)</th>
<th>Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control$^a$</td>
<td>4.0±0.3</td>
<td>58500±583</td>
</tr>
<tr>
<td>Control$^b$</td>
<td>3.4±0.5</td>
<td>223750±2237</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.1±0.1$^+$</td>
<td>73800±738$^+$</td>
</tr>
<tr>
<td>Bracken</td>
<td>2.3±0.0$^+$</td>
<td>55000±550$^+$</td>
</tr>
<tr>
<td>Shikimate</td>
<td>3.6±0.2</td>
<td>204000±2040</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.

a - Experimental control from 28 day study
b - Historical control from Mitruka and Rawnsley (1977).

+ - value significantly lower than controls a and b (p < 0.05)
(Using the Student's t-test)
The results of the mean cell volume (MCV) and mean cell haemoglobin (MCH) are above normal historical values and may indicate errors in the estimation of the total erythrocyte counts, and therefore the reticulocyte counts derived from them. Although presented in Table 3.6 no conclusions are drawn from them.

The total leucocyte counts are also presented but intrusive pathology (3.10.4) renders the control group redundant and the shikimate group has been abandoned for comparative purposes.

The quercetin-fed rats had reduced haemoglobin and packed cell volume, leucopenia, and thrombocytopenia.

Table 3.7 Mean Cell Volume (MCV), Mean Cell Haemoglobin (MCH), and Mean Corpuscular Haemoglobin Concentration (MCHC) after 28 days, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets

<table>
<thead>
<tr>
<th>Groups</th>
<th>MCV (fm)</th>
<th>MCH (pg)</th>
<th>MCHC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^a)</td>
<td>81.1±0.1</td>
<td>28.3±0.2</td>
<td>34.0±0.2</td>
</tr>
<tr>
<td>Control(^b)</td>
<td>53.8±0.1</td>
<td>16.3±0.1</td>
<td>30.8±0.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>82.9±0.7</td>
<td>28.0±0.1</td>
<td>33.8±0.2</td>
</tr>
<tr>
<td>Bracken</td>
<td>78.1±0.4</td>
<td>25.4±0.1</td>
<td>32.5±0.2</td>
</tr>
<tr>
<td>Shikimate</td>
<td>73.3±0.7</td>
<td>20.0±0.3</td>
<td>27.2±0.4</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
\(^a\) - Experimental control from 28 day study
\(^b\) - Historical control from Mitruka and Rawnsley (1977).
Table 3.8 Absolute Differential Leucocyte Counts after 28 days, of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets

<table>
<thead>
<tr>
<th>Groups</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control a</td>
<td>180±60</td>
<td>75±30</td>
<td>1500±180</td>
<td>105±30</td>
<td>13200±150</td>
</tr>
<tr>
<td>Control b</td>
<td>350±11</td>
<td>200±15</td>
<td>2420±20</td>
<td>230±20</td>
<td>7540±826</td>
</tr>
<tr>
<td>Quercetin</td>
<td>120±47</td>
<td>26±13</td>
<td>281±87</td>
<td>46±13</td>
<td>6231±134</td>
</tr>
<tr>
<td>Bracken</td>
<td>932±186*</td>
<td>80±25</td>
<td>2113±229</td>
<td>249±62</td>
<td>2734±248*</td>
</tr>
<tr>
<td>Shikimate</td>
<td>378±70</td>
<td>70±28</td>
<td>896±210</td>
<td>140±28</td>
<td>12460±280</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
a - Experimental control from 28 day study
b - Historical control from Mitruka and Rawnsley (1977).
* - value significantly higher than controls a and b (p < 0.05)
+ - values significantly lower than controls a and b (p < 0.05)
(Using the Student's t-test)

The bracken-fed rats had leucopenia and thrombocytopenia, while the shikimate group had reduced haemoglobin.

The differential absolute leucocyte counts revealed an eosinophilia and leucopenia for the bracken-fed rats and a neutropenia for the quercetin-fed rats.

Examination of the blood films revealed that in the control group there was a mild anisochromasia, mild anisocytosis and small platelet clumps seen occasionally across the film. The erythrocytes contained no inclusion bodies.

The predominant leucocyte was the mature lymphocyte, identified by the large nucleus and the corresponding small cytoplasm. There were occasional immature lymphocytes with a large nucleus and practically no cytoplasmic space.

The monocyte was rare, but was kidney-shaped and had a vacuolated cytoplasm.
The neutrophil was evident as a cell with a pale blue to colourless cytoplasm containing small granules, which were few in number. The nucleus was lobed but less clearly defined than in the eosinophil. A few young neutrophils were identified by the presence of "doughnut" ring nuclei.

The eosinophils were larger than neutrophils, had a bilobed nucleus, and contained deeply acidophilic-staining granules.

The basophils were very rare but identifiable by their dark-staining granules.

The blood film of the quercetin-fed rats revealed a marked polychromasia, with clusters of small platelets seen throughout the film. The predominant leucocyte was the mature lymphocyte. The other leucocytes were identified but revealed no striking features different from the normal.

The shikimate-fed rats showed increased polychromasia, increased microcytosis, anisocytosis and hypochromasia. The dominant leucocyte was the lymphocyte. There was an increase in the number immature neutrophils. The platelets were larger than in the control films.

The polychromasia of the bracken-fed rats was less marked than in the shikimate group. There was microcytosis and hypochromasia with an accompanying anisocytosis and poikilocytosis. There was a predominance of small erythrocytes. Rouleaux formation was evident, the platelets were small sized and the predominant leucocyte was the mature lymphocyte.
3.11 Discussion

3.11.1 The Metabolism of Cyanide

The investigation of the bracken-induced ataxia in rats was carried out on the premise that other consituents of bracken apart from but probably in addition to thiaminase may play a role in the induction of the ataxic syndrome. The main bracken constituent thought to induce this was cyanide derived from the cyanogenic glycoside, prunasin which in some bracken samples may be as high as 50 mg per 100 g dry weight of bracken (Moon and Raafat, 1951b). The other bracken constituents, shikimate and quercetin are not thought to induce ataxia but the in vivo ataxic activity of the "SF Factor" (Konishi, 1984) in the equine suggests that thermostable factors capable of inducing ataxia also exist in bracken. Quercetin and shikimate were studied for comparative purposes.

To this end a sensitive fluorimetric assay method (Hanker et al., 1958) was used to determine the serum and urinary levels in the various test and control groups. It has the advantage of not using benzidine which is a bladder carcinogen employed in the method of Aldridge (1944), it has a comparable sensitivity (Pettigrew and Fell, 1972), and the use of serum rather than whole blood ensures that the cyanide so determined is the active fraction of cyanide as against whole blood where erythrocytic enzyme, thiocyanate oxidase may reconvert some thiocyanate into cyanide (Goldstein and Reiders, 1953). The activity of thiocyanate oxidase is probably responsible for the presence of cyanide in the serum of control animals and human subjects not ingesting cyanide directly. Thiocyanate is a normal constituent of human saliva and may be converted to cyanide by thiocyanate oxidase (Smith et al., 1963; Wells et al., 1972). The method used here is however less sensitive than the chloramine-T method of Boxer and Rickards (1960) which showed linearity over the range 0.005 to 0.40 ug/ml cyanide with a coefficient of variation of 1.5%. The possibility of interfering substances has been corrected for by the recovery studies, but in contrast to the method of Aldridge (1944) where the coefficient of variation as applied to by other workers (Osuntokun, 1973) ranged from 32.2% to 375.4% it may be considered a comparatively sensitive method. Furthermore, by using the excitation and emission wavelengths specified in 3.9.6 the specificity of the method has been improved.
The bracken-fed rats had significantly higher cyanide levels in their sera and this may be a direct consequence of the failure of rhodanese enzyme to convert cyanide to thiocyanate. Cyanide retention by bracken-fed animals had been previously reported by Moon and Raafat (1951b) in sheep and bullocks but they concluded that cyanide was not a significant constituent of bracken, probably due to difficulties with estimating the amount of cyanide exhaled in the breath, and also probably due to the fact that gastroenteritis which is a consistent feature of bracken toxicity is not usually found in cyanide toxicity. However these rats had significantly higher cyanide levels, due in the main to the failure of the rhodanese enzyme in the bracken-fed rats. The rhodanese activity of the control rats at 0.33 umols thiocyanate/min/mg protein is comparable to the value of 0.49 umols thiocyanate/min/mg protein determined by Osuntokun (1970) although his rats ranged in age from 6 to 18 months. The coefficient of variation in this study is to be compared to the range of 32.0% to 57% for various organs determined by Osuntokun (1970).

The factors responsible for the impaired activity of rhodanese may include the reduced availability of sulphur donor compounds such as methionine which occurs in cyanide toxicity (Tewe, 1985). Also the thiaminase enzyme may contribute by destroying thiamine, but the overall effect would be to limit the availability of co-factors or sources of sulphur for the operation of rhodanese. In addition to being a substrate for rhodanese, cyanide may also act as an inhibitor of rhodanese, an inhibition which may also be brought about by other substrates such as nicotine (Schievelbein et al., 1969).

The effect of the failure to detoxify cyanide is the accumulation of cyanide which then contributes to the ataxic syndrome.

In addition, the myeloperoxidase enzyme (MPO) found in polymorphonuclear cells (PMNs) may play a role in the production of cyanide. In an inflammatory response as occurred in the liver of bracken-fed rats (3.10.4) and which is discussed later (3.11.4), the azurophil granules of the PMNs may release MPO, which in combination with hydrogen peroxide and chloride may react with some amino acids like glycine to yield hydrogen cyanide (Stelmaszynska and Zgliczynski, 1981). The hydrogen peroxide may be produced as part of a free-radical component of bracken toxicity which is
commented upon later (3.11.4). However, this additional source of cyanide generation in bracken-intoxicated rats must be viewed against the background that in bracken-intoxicated animals a wide range of amino acids is depleted (Tewe, 1985), and glycine may not necessarily be available.

The main product of cyanide detoxification is thiocyanate. The enhanced activity of rhodanese in the shikimate- and quercetin-fed rats is reflected in the higher levels of urinary thiocyanate in these two groups. The serum levels of thiocyanate were not significantly different and this may be explained in part by the non-linear pattern of thiocyanate biotransformation in the rat. Thiocyanate is absorbed from the small intestine into the blood, where some may be converted into cyanide by the erythrocytic thiocyanate oxidase. Thiocyanate is then secreted from the blood into the stomach where some may be sequestered depending on the amount of food available in the stomach, and where it may enter into various reactions, including the catalysis of N-nitrosation. Any thiocyanate left may be transported down the gut and be absorbed from the small intestine and the cycle goes on. In the rat there is a gastro-intestinal re-circulation of thiocyanate and unlike in the human, the rat salivary gland does not secrete thiocyanate (Logothetopoulos and Myant, 1956; Okoh, 1978). This non-linear biotransformation of thiocyanate causes wide variability in serum thiocyanate levels. This notwithstanding the value of 4.2 ug/ml for the control rats is comparable to the value of 5.3 ug/ml obtained by Ermans et al. (1973) for rats on a six-week experiment. In addition to the non-linear biotransformation, the diet of the rats also affects the serum thiocyanate levels. Dams on a rat chow diet had a serum thiocyanate value of 8.17 ug/ml to 9.46 ug/ml while those on a Remington diet had values of 2.49 ug/ml to 2.51 ug/ml (Kreutler et al., 1978).

The urinary levels of thiocyanate were considered more representative of the body load. The urinary thiocyanate level of 1.6 ug/mg creatinine (= 2.4 ug/ml) is lower than the value of 5.8 ug/ml determined by Smith and Foulkes (1966) but the rats in this study had various kidney lesions which may have influenced this (see 3.11.4) although these are considered to be related to the ad libitum pattern of feeding.

These differences are reflected in the differing coefficients of variation between thiocyanate determined in the two biological fluids. Indeed when determined by other methods by other workers the differences
between the two were also remarkable. For human plasma the technique of Pettigrew and Fell (1972) gave a coefficient of variation of 69% (Gardner et al., 1984) while the method of Aldridge (1944) gave a coefficient of variation of 34% for human urinary thiocyanate (Osuntokun, 1973).

Although this method is an improvement on previous methods using ferric chloride, it nevertheless suffers the disadvantage of a reduced specificity due to other compounds such as cyanide.

It ought, in addition to the foregoing, be borne in mind that other pathways involving cyanide detoxification apart from the rhodanese-thiocyanate pathway exist, although they are less important (Oke, 1973). Some cyanide may also be exhaled as hydrocyanic acid and carbon dioxide, and nitrite also has an influence in reducing the available body pool of cyanide (Johnson and Isom, 1985).

3.11.2 Nitrite Levels

The nitrite levels were determined because of the ability of nitrite to act as an antidote of cyanide toxicity. However, when the level of nitrite in the system gets too high it may induce ataxic syndromes and abnormalities in the brain activity of rats (Behroozi et al., 1972). The bracken-fed rats and the quercetin-fed rats had significantly high urinary levels of nitrite.

The method of Montgomery and Dymock (1961) is very sensitive and easily reproducible. The application of the method by Bassir and Maduagwu (1978) was more sensitive but had a higher coefficient of variation due to the nature of the crude mixtures they were analysing. They had a sensitivity of 0.2 µg/ml compared to 0.3 µg/ml in this study, but their coefficients of variation ranged from 20 to 58%. However, recovery of added nitrite was as high as 98.3 ± 0.7%.

The origin of the significantly high levels of nitrite in the urine of the quercetin- and bracken-fed rats may be due to the reduction of nitrate, a reaction catalysed by micro-organisms in the mammalian alimentary tract or by tissue nitrate reductase enzyme (Ayanaba and Alexander, 1973; Omura, 1959).
Nitrate is an ubiquitous component of foods being present in fruits, vegetables and potable water (Hill et al., 1973; White, 1975). The nitrate content of the bracken and quercetin diets was not determined, neither was the amount in the local water supply. Although the diet is the most probable source of nitrate and hence nitrite, it is by no means the only one. The in vivo synthesis of nitrate appears to be a normal mammalian process as rats on a low nitrate diet excreted more urinary nitrate than could be accounted for by ingested nitrate alone (Green et al., 1981). The metabolism of quercetin (a constituent of bracken) is dependent in part on the nature of the gut flora and it is possible that the gut flora is transformed in such a way as to promote the growth of nitrite-yielding bacteria in the gut of quercetin- and bracken-fed rats. This does not exclude the role of tissue nitrate reductase or the possibility that the nitrite so produced may be a breakdown product of N-nitroso compounds. The bacterial breakdown of N-nitroso compounds may yield nitrite as one of its products (Rowland and Grasso, 1975).

Although the origin of nitrite is open to speculation, its significance is clear. It could be implicated along with cyanide in the ataxic syndrome observed in bracken-fed rats. Furthermore, it may play a role in the nitrosation of various secondary and tertiary amines to form N-nitroso compounds, but this is further examined in Chapter Four. Clinical evidence supports the view that nitrite may induce symptoms similar to those of cyanide in domestic animals (Blood et al., 1979).

The bracken-induced ataxia of rats may therefore be a multifactorial phenomenon, of which cyanide and nitrite are two of the possible aetiologcal factors, including the thiaminase enzyme which may act to predispose the animals to the toxic effects of cyanide.

3.11.3 Haematology

The haematological indices are suggestive of the ability of the test substances, shikimate and quercetin and bracken the composite from which they are isolated to inflict toxic injury on the erythropoietic system. The haemoglobin levels and the haematocrit were depressed relative to the experimental and historical controls. In addition there was a thrombocytopenia in all the three test groups but even though in the
shikimate group it was not statistically lower than the control, it nevertheless revealed a biological downward trend. These are indicative of bone marrow depression by these test compounds. However, the MCV and MCH values calculated from the observed data were higher than the historical values and may indicate errors in the estimation of the erythrocyte number and therefore of the reticulocyte numbers estimated from them. Therefore the comments here do not include any references to or any inferences from these indices.

The polychromasia observed from the smears is indicative of the presence of cells of varying ages and it may thus be inferred that there is a regenerative process in these three groups. This view is further buttressed by the large number of megakaryocytes which are the precursor cells of platelets especially in the bone-marrow of the bracken-treated rats. In addition the bone marrow in the three test groups revealed red filled cavities and little or no fat spaces and it may be inferred that this is regenerative. This inference must however be viewed with caution as it is based on a single time-point observation of what is essentially a dynamic process. However, it is pertinent to draw attention to the extramedullary haemopoiesis which was particularly prominent in the spleen of the bracken-fed rats. Though this process was going on in the quercetin- and shikimate-fed rats, they were judged to be no more than would be expected in a normal (control) rat. Thus it may be that at the time of sampling, the bracken-fed rats were at a further stage of regeneration than the two isolates.

The results for the bracken-fed rats are in accordance with the previously published observations on bracken toxicity, but in the case of the quercetin-treated rats, they are at variance with a previous study in which a 1% (w/w) quercetin diet did not induce any haematological changes in albino rats at 70, 250 and 410 days respectively (Ambrose et al., 1952).

The Rouleaux formation observed in the bracken-fed rats is evidence of the clinical illness which was observed before the experiment was terminated, and may indicate a greater tendency of the blood to clot more easily in these rats.

The differential leucocyte counts indicate an eosinophilia and lymphocytopenia in the bracken-fed rats. These conclusions were reached because in comparison with both experimental and historical controls, these
changes were still valid. To further buttress the view that these changes were genuine is the infiltration of eosinophils into the lungs and spleen of the bracken-fed rats. This may be as a result of a hypersensitivity response. The absence of an accompanying monocytosis, however, renders this assertion questionable, but monocytosis in the rat is a very rare phenomenon. Recently, two glucuronyl pyranosides called braxin A1 and braxin A2 capable of eliciting the release of vasoactive amines from rat peritoneal mast cells were isolated from bracken (Saito and Mochizuki, 1986). The vasoactive amine, histamine is a mediator of immune responses in animals. The release of histamine and heparin in bracken-intoxicated cattle had been previously reported as part of a possible immune response in bracken toxicity (Evans, 1968; Leach and Evans, unpublished data). This raises the issue of the type of immune reaction which this might be. Eosinophils are usually mobilised in type I (anaphylactic) reactions where as a result of re-exposure to a particular antigen, IgE attached to mast cells causes them to release mediators like histamine, bradykinin and Slow Reacting Substance of Anaphylaxis (SRS-A) whose main actions are on the smooth muscle, vascular endothelium and on mucus secretion. As a result, oedema of the nose and throat are observed as clinical signs. The eosinophils are attracted by Eosinophil Chemotactic Factor A (ECF-A) and the eosinophils release histaminase and other inhibitors which neutralise the anaphylactic response. That this explanation might be plausible is reflected in the earlier studies of Fletcher (1944) who reported a laryngitic form of bracken poisoning in cattle characterised by swelling of the throat region and roaring (1.3.1). This might be the first recognised report of an anaphylactic response in bracken toxicity.

In considering the presence of eosinophils and the validity of the immunological interpretation, it is pertinent to bear in mind that no previous contact with bracken was observed before the feeding of bracken in the 28 day study, and these rats besides had an intrusive pathology, so that the eosinophilia may be considered as part of a "sympathetic response" by the eosinophils. Their presence in the spleen, however, would tend to rule this possibility out.
3.11.4 Histopathology

The histopathological lesions in the lungs of both the control and test animals are evidence of intrusive pathology not related to compound treatment. The control and shikimate test groups have reacted most markedly as shown by the lymphocyte counts, hence they were considered untenable for comparative purposes and while the shikimate group was abandoned for that reason, historical controls have been substituted for the experimental controls.

The kidneys showed evidence of deposits in the collecting ducts in the cortico-medullary zone which may be diagnosed as nephro-calcinosis. This change is probably responsible for the other changes such as hydropic change and focal tubular nephritis in the proximal tubules, and occasional thickening of the parietal surface of the Bowman's Capsule. These being observed in both test and control groups of rats were not attributable to intrusive pathology, but are rather a consequence of the ad lib. feeding method in these essentially young rats, with a resulting imbalance in calcium : phosphorus ratio and the deposition of calcium in the collecting ducts. This feature had been observed in the University of Surrey strain of Wistar Albino rats. However, this may be reflected in the lower urinary thiocyanate levels of the control group compared to literature values. Their generalised occurrence is, however, thought to indicate a particular pattern of metabolism for this age of rats rather than a compound-related pathology.

The liver lesions in the bracken-fed group are however thought to be a compound-related effect which may have arisen as a result of free-radical damage of the hepatocytes resulting in the invasion of the liver by inflammatory cells, both polymorphonuclear cells and mononuclear cells, although the overall picture was of predominantly mononuclear cell infiltration. Bracken toxicity affecting the liver had been reported previously (Hirono et al., 1984a) but this was an isolated report.

The predominance of round cells would suggest a chronic inflammatory response to free-radical induced damage. It may also represent a delayed hypersensitivity (or type IV) immune response to cells which now damaged are recognised as foreign. The interpretation is buttressed by the low lymphocyte numbers in the differential absolute counts of the bracken-treated group, which perhaps suggests that these cells are sequestered in
the tissues as, for example, in the liver. That bracken toxicity is mediated via free-radicals has been suggested by the studies of Pamucku et al. (1970) and Evans (1985). Apart from free-radicals, however, lymphokines released from macrophages may cause local tissue ischaemia and necrosis. The pattern of periportal tissue necrosis in the liver of the bracken-fed rats is in accordance with this interpretation. It is probably a general hepatic response to toxic injury rather than a specific toxic lesion of bracken. This sort of response by the liver has been observed in rats poisoned by the pyrollizidine alkaloids of Crotalaria, aflatoxins B and G and dimethyl-nitrosamine (McLean et al., 1965).

The other possible consequence of the involvement of free-radicals has been briefly mentioned earlier. The polymorphonuclear cells released in the early part of the immune response release myeloperoxidase which may play a role in the generation of cyanide (vide supra).

3.11.5 Conclusion

In conclusion, therefore, the picture that emerges from these studies is of a failure by bracken-intoxicated rats to detoxify cyanide mainly as a result of the failure of the rhodanese enzyme, a failure which may be brought about by a shortage of sulphur-donor compounds or even as a result of poisoning by the cyanide which acts as its substrate. The consequence of this failure is the accumulation of cyanide which is a cause of ataxia in animals and humans. In addition, though nitrite may act as an antidote of cyanide, its toxicity is similar to that of cyanide if it accumulates to very high levels as was the case in the bracken-intoxicated rats. In the event, it would appear that there is a substantial case for implicating nitrate and nitrite in bracken toxicity. Almost uniformly, gastro-enteritis is found in all cases of nitrate-nitrite toxicoses but not in cyanide toxicoses (Hibbs, 1979). The pattern in bracken-intoxicated cattle shows that gastro-enteritis is a consistent feature of bracken toxicity. While cyanide and nitrite may be causes of bracken-induced ataxia in animals, the role of thiaminase as a cause is still valid.

On the other hand, the detoxification of cyanide in quercetin- and shikimate-fed rats was enhanced resulting in very high levels of thiocyanate in the urine of these rats.
The haematological observations reveal a bone marrow depressant effect by bracken and its constituent isolates, quercetin and shikimate, while the liver lesion in the bracken-intoxicated rats was attributable to an immunologically induced injury probably in response to free-radical attack on the hepatocytes.

The implications of high nitrite and thiocyanate in the toxicology of bracken with specific reference to N-nitrosation will be examined in the next Chapter.
CHAPTER FOUR

N-NITROSATION STUDIES ON BRACKEN, QUERCETIN AND SHIKIMATE
4.1 Introduction

The studies described in this chapter were designed to investigate the possible role of N-nitrosation in bracken-induced carcinogenesis.

In the previous experiments (Chapter Three), bracken-fed, and to a lesser extent quercetin-fed rats excreted in their urine, large amounts of nitrite. The nitrite, together with cyanide derived from prunasin could be responsible for the bracken-induced ataxia observed in bracken-fed rats. In addition, nitrite and other oxides of nitrogen are known to form very toxic and carcinogenic N-nitroso compounds with a group of nitrosatable compounds which include secondary and tertiary amines, amides, N-alkylureas, N-alkylcarbamates, cyramides, guanidines, amidines, hydrazines, hydrazones, hydrazides, 3-methylindole (skatole), indole-3-acetic acid and N-acetyl tryptophan (Mirvish, 1975). It is essential to differentiate between N-nitroso compounds and C-nitroso compounds which are formed by the reaction of nitrites and some phenols and are generally non-carcinogenic or only very weak carcinogens (Mirvish, 1975).

4.2 The Toxicology of N-Nitroso Compounds

N-nitroso compounds have a wide spectrum of toxicological effects, including acute and chronic cellular injury, mutagenesis, teratogenesis and carcinogenesis (Magee and Swann, 1969; Shank, 1975).

Their toxic effects depend on their chemical nature - the nitrosamines are usually activated metabolically in the liver to a reactive species whose toxic effects are usually in the liver with a few exceptions such as dibutylnitrosamine which selectively and specifically attacks the urinary bladder. The nitrosamides on the other hand tend to decompose to species which attack the organs of rapid cell turnover such as the lymphoid tissue, the haematopoietic tissue and the gastro-intestinal tract (Magee and Swann, 1969; Lobl, 1972; Shank, 1975).
4.3 Sources of Nitrosatable Compounds

Various chemical compounds are capable of reacting with nitrite to form N-nitroso compounds (Section 4.1). With specific regard to bracken, bracken-intoxicated calves had increased levels of circulating heparin, histamine and serotonin (Evans and Howell, 1962; Mason, 1965; Leach and Evans, unpublished data, cited by Evans, 1970). In addition, pterolactam, isolated from bracken contains a nitrosatable group. These provide a source of nitrosatable compounds. The nitrosatability of serotonin, a tryptophan metabolite, has been demonstrated (Gatehouse and Wedd, 1983).

This raises the issue of the role of tryptophan metabolites and tryptophan metabolism in bracken toxicity. Some tryptophan metabolites like indole, indoleacetic acid, 8-hydroxyquinaldic acid and xanthurenic acid are tumour promoters, while others like 3-hydroxykynurenine and 3-hydroxyanthranilic acid are bladder tumour promoters and are also mutagenic (Hill, 1980).

It may therefore be of interest to examine tryptophan metabolism in the toxicology of bracken in view of the nitrosatability of metabolites like serotonin and the tumour promoting ability of others like xanthurenic acid.

The work of Dalgleish (1955) showed that thiamine is required as a co-factor for the enzyme tryptophan pyrrolase responsible for the conversion of tryptophan to N-formylkynurenine, which is the first step in the kynurenine pathway of tryptophan metabolism. It may thus be possible to envisage a situation such as occurs in bracken toxicity where avitaminosis $B_1$ occurs, and the kynurenine pathway is impaired in favour of the indole pathway of tryptophan metabolism, leading to the production of potentially nitrosatable compounds.

Although tryptophan metabolism had been inconclusively studied in bracken-intoxicated cows (Pamukcu et al., 1959; Pamukcu and Bryan, 1979) the use of a different animal model in the form of a monogastric provides an opportunity to re-examine an old subject.
Figure 4.1 The Formation of N-Nitroso Compounds

Secondary amine + Nitrite \[ \rightarrow \] Nitrosamine

\[ R^1 \text{NH}_2 + HNO \rightarrow R^1 \text{NH} \text{CR}^2 \text{NO} \rightarrow R^1 \text{N} \text{CR}^2 \text{NO} \]

(Lobl, 1972; Shank, 1975; Wogan, 1975)

\[ R^1, R^2 \text{ may be substituted or unsubstituted aryl or alkyl compounds.} \]
The Indole and Kynurenic Pathways of Tyrosine Metabolism

Key:

a = transaminase
b = transaminase
c = ammonia dehydrogenase
d = tyrosine decarboxylase
e = amiontransferase
f = ammonia oxidase
g = tyrosine decarboxylase
h = tyrosine hydroxylase
i = tyrosine hydroxylase

Figure 4.2
4.4 Factors Affecting N-Nitrosation

The formation of N-nitroso compounds requires that nitrite and a nitrosatable compound be present together at the same site. In addition, acid pH facilitates their formation as do thiocyanate, halide ions, kaempferol, quercetin, catechin, and naringenin which are catalysts of N-nitrosation (Wogan and Tannenbaum, 1975; Mirvish, 1975; Boyland et al., 1971; Mirvish et al., 1975; Pignatelli et al., 1980, 1982).

Conversely, alkaline or neutral pH will inhibit the formation of N-nitroso compounds except in the presence of formaldehyde (Shank, 1975) as will caffeic, ferulic, and chlorogenic acids, gallic acid, fisetin, trolox, butylated hydroxyanisole, ascorbate, butylated hydroxytoluene and tert-butyl hydroquinone (Wogan and Tannenbaum, 1975; Mirvish et al., 1975; Astill and Mulligan, 1977; Kuenzig et al., 1984).

In addition to these chemical factors, some bacteria enhance N-nitrosation even at neutral pH. Thus in the human achlorhydric stomach, in the infected urinary bladder and in the colon, bacteria play a role in in vivo N-nitrosation (El-Merzabani et al., 1979; Hill, 1979).

4.5 Objectives of the Study

The objectives of this study are to determine:

i) the possible role of N-nitrosation in bracken-induced carcinogenicity.

ii) what contributions (if any) quercetin and shikimate might make to such a process.

iii) if there is an alteration of tryptophan metabolism in bracken-fed rats.

iv) if quercetin and shikimate may also induce such alterations in tryptophan metabolism.

v) if such alteration may be implicated in bracken carcinogenicity.

4.6 The Strategy of the Study

Some of the factors affecting N-nitrosation were determined in a 90-day study. These include nitrite levels in the urine of rats fed 10% (w/w) bracken, 1% (w/w) quercetin, 1% (w/w) shikimate or control diets. In
addition, thiocyanate, a catalyst of N-nitrosation was determined in the urine and terminally in the serum of these rats.

Tryptophan metabolites like xanthurenic acid which is known to be an intermediate in the kynurenine pathway of tryptophan metabolism and is a known bladder carcinogen (Allen et al., 1957) as well as kynurenic acid were determined in the urine of these rats.

The indole pathway was examined by assaying for total 5-hydroxyindole using 5-hydroxytryptamine as a marker (Udenfriend et al., 1955; Varley et al., 1976).

The comparison of the shikimate- and quercetin-fed rats to bracken-fed rats is important not only on the basis of a need for determining how they, as bracken isolates compare with the parent compound, but in its own right quercetin is a catalyst of N-nitrosation. In addition, as a mutagen it may enhance the growth of intestinal microflora which could influence in vivo N-nitrosation. The role of shikimate in N-nitrosation is not yet explored but its metabolism is carried out in part by the gut flora (Brewster et al., 1978) hence the need to determine how this may influence N-nitrosation.

4.7 Materials and Methods

4.7.1 Animals

Forty male Wistar-albino (W/A) rats (University of Surrey Animal Unit) were divided into four groups of 10 per group as previously described.

4.7.2 Feedstuffs and Diets

Bracken (10% w/w), quercetin (1% w/w) and shikimate (1% w/w) diets were prepared as previously described.

4.7.3 Chemicals and Reagents

These were of analytical reagent grade, unless otherwise stated.
4.7.4 Sample Collection

Urinary samples were collected at weeks 4, 8, and 13 by putting the rats into metabole cages and collecting 24 hour urine. The volume and pH of each urine sample were noted and the urine sample from each group divided into three. One portion was used for the determination of creatinine, nitrite, thiocyanate and total 5-hydroxyindoles.

The second portion was acidified with sulphamic acid and used for N-nitroso compound studies. The sulphamic acid stabilises the urine by suppressing bacterial growth, inactivates nitrosating agents, and prevents decomposition of N-nitroso compounds. The third portion was acidified with 2M hydrochloric acid (2 ml per 10 ml urine aliquot) for the determination of xanthurenic and kynurenic acids.

At week 13 when the study was terminated each rat was anaesthesised with anaesthetic ether (May and Baker Ltd., England) and terminally exsanguinated via the posterior vena cava. The blood collected was left to clot in plain glass tubes for the collection of serum which was used for the determination of thiocyanate.

The gut contents were collected by separately tying off the stomach, intestine and caecum and flushing out with distilled water. Each portion was thoroughly dried on filter paper and weighed out in 1 g portions. Sulphamic acid was added to each portion. All the samples were stored at -12°C until analysed usually within three days.

4.7.5 Determination of Creatinine

Urinary creatinine was determined as described in 3.9.9.

4.7.6 Determination of Urinary Nitrite

Urinary nitrite was determined as described in 3.9.8.
4.7.7. Determination of Urinary and Serum Thiocyanate

Urinary and serum thiocyanate were determined as described in 3.9.5.

4.7.8 Determination of N-Nitroso Compounds in Urine and Gut Contents

The urine sample containing sulphamic acid was filtered, sodium chloride (BDH Chemicals Ltd., Poole, Dorset, England) was added (10% w/v) and the solution exhaustively extracted with ethyl acetate (May and Baker Ltd., Manchester, England) using 10.0 ml ethyl acetate per ml urine on each occasion using flat bottomed flasks. The gut contents were extracted with 100 ml ethyl acetate per 1 g of gut content by shaking in flat bottomed flasks.

To each ethyl acetate extract of either the gut content or urine sample, an equal volume of 3% hydrogen bromide (BDH Chemicals Ltd., Poole, England) in glacial acetic acid (BDH Chemicals Ltd., Poole, England) was added and left at room temperature for 15 minutes. A 20 ul aliquot was added to 2.5 ml sulphanilic acid made up of 27.2 g potassium hydrogen sulphate (Hopkin and Williams Ltd., Essex, England) and 3.46 g sulphanilic acid (BDH Chemicals Ltd., Poole, Dorset, England) made up to 1 L with distilled water. After 10 minutes, 2.5 ml of NEDH, 0.04% (w/v) in distilled water was added. The pink colour was allowed to develop for 20 minutes and the absorbance was determined using a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England) at 550 nm. To ensure that residual nitrite was not mistaken for N-nitroso compounds the blank was made up of extract minus the hydrogen bromide (Eisenbrand and Preussmann, 1970).

The standard curve was prepared by using sodium nitrite in distilled water according to the method of Montgomery and Dymock (1961) as described in section 3.9.8. The assay was linear between $1.00 \times 10^{-4}$ and $2.00 \times 10^{-2}$ mg/ml.

4.7.9 The Nitrosation Assay Procedure (NAP)

The NAP was carried out as described by Coulston (1980) and modified by Gillatt et al. (1984).
To each 1 ml of urine, or 1 g of gut content acidified with sulphamic acid, were added sodium chloride, 0.5 mg, plus sufficient 0.05 M KCl-HCl buffer containing 10 mM potassium thiocyanate to bring the pH to pH 3. Sodium nitrite was added to give a concentration of 40 mM in the final volume. It was incubated at 37°C for 3 hours, and at the end excess sulphamic acid was added to remove residual nitrite which could give false positive results. Extraction of N-nitroso compounds was done with ethyl acetate and determined as already described.

The determination of N-nitroso compounds was based on the method of Eisenbrand and Preussmann (1970) with the modification that the method of Montgomery and Dymock (1961) was used for the detection of nitrite rather than the Greiss-Ilosvay reagent.

The method is rapid, sensitive and specific for N-nitroso compounds. It does not detect related compounds such as N-nitramines, N-nitramides, C-nitro and C-nitroso compounds, oximes and alkyl nitrates (Johnson and Walters, 1971).

The reaction is based on the cleavage of the N-nitroso group by hydrogen bromide in the presence of glacial acetic acid.

**Figure 4.3 Cleavage of the N-Nitroso Bond by Hydrogen Bromide**
(The principle of the determination of N-nitroso compounds, Eisenbrand and Preussman, 1970)

\[
\begin{align*}
\text{R}^1\text{R}^2\text{N} = \text{O} + \text{HBr} \rightarrow & \text{Glacial acetic acid} \\
\left[\begin{array}{c}
\text{R}^2 \\
\text{R}^1 \end{array}\right] \text{Br}^- + \text{O} = \text{N} \rightarrow \text{NO}^+ + \text{Br}^-
\end{align*}
\]

The NO\(^+\) thus released is captured by diazotization of sulphanilamide and the diazonium ion which results is coupled with NEDH and can be measured spectrophotometrically at 550 nm.
For the assay system to work well, it has to be as anhydrous as possible, and an average recovery of 99.5% could be obtained, so that in the determination of N-nitroso compounds each nitrite molecule could be considered equivalent to one molecule of N-nitroso compound (Newton, 1978).

4.7.10 Determination of Xanthurenic and Kynurenic Acids

The portion of urine acidified with hydrochloric acid was diluted 1 : 5 with distilled water and 4.0 ml aliquots were used for the duplicate determination of xanthurenic acid and kynurenic acid by pouring the urine samples down a 3.0 cm column of Dowex 50-W ion exchange, acid form resin in a bedding of cotton wool. The resin column was prepared prior to use by washing successively with 5.0 ml 2 M hydrochloric acid and 10.0 ml distilled water. The eluate and the first 10.0 ml portion of distilled water from the column were discarded. The last 25.0 ml portion was used for the determination of xanthurenic acid and kynurenic acid.

Xanthurenic acid was determined by adding 1.0 ml saturated sodium hydroxide to 1.0 ml of the eluate and centrifuged at 3,000 g for 5 minutes in a bench top centrifuge to give a clear solution whose fluorescence was determined in a Perkin-Elmer fluorescence spectrophotometer, Model LS-5 (Perkin-Elmer Ltd., Bucks., England), with the excitation wavelength at 370 nm and the emission wavelength at 515 nm.

Kynurenic acid was determined by adding 1.0 ml 18 M sulphuric acid to 1.5 ml of the eluate. After leaving to cool the fluorescence was determined at an excitation wavelength of 365 nm and an emission wavelength of 440 nm using a Perkin-Elmer fluorescence spectrophotometer, Model LS-5 (Perkin-Elmer Ltd., Bucks., England).

The blank in each case was made up of distilled water passed through the column and treated as above (Bender et al., 1977).

Xanthurenic and kynurenic acids (Sigma Chemical Company Ltd., Poole, Dorset, England) were used for the preparation of the standard curve. The xanthurenic acid assay was linear over the range 1.0 x 10⁻⁵ to 1.0 x 10⁻² mg/ml while the kynurenic acid assay was linear over the range 1.0 x10⁻⁵ to 1.0 mg/ml. Recovery studies were carried out by adding quantities of
commercial xanthurenic acid and kynurenic acid to aliquots of various urine samples and processing them as described above. The percentage recovery determined in each case ranged from 96% to 104% for xanthurenic acid and from 91% to 103% for kynurenic acid.

4.7.11 Determination of Total 5-Hydroxyindoles

To duplicate 1.0 ml aliquots of urine were added in succession, 1.0 ml distilled water, 1.0 ml 1 g/L 1-nitroso2naphthol in ethanol and 1.0 ml nitrous acid reagent made up of 0.2 ml 25 g/L sodium nitrite in distilled water and 5.0 ml 1 M sulphuric acid. The nitrous acid reagent was always freshly prepared. After 10 minutes, it was shaken twice with ethyl acetate, 5.0 ml on each occasion. The ethyl acetate layers were discarded and the aqueous layer was centrifuged at 3,000 g for 5 minutes in a bench-top centrifuge. The absorbance of the supernatant was determined in a Cecil CE 292 digital UV spectrophotometer (Cecil Instruments Ltd., Cambridge, England) at 540 nm against a blank containing water instead of urine (Udenfriend et al., 1955; Varley et al., 1976).

The standard curve for total 5-hydroxyindoles was prepared with 5-hydroxytryptamine (Sigma Chemical Company Ltd., Poole, Dorset, England). The assay was linear between $2.2 \times 10^{-3}$ and $17.6 \times 10^{-3}$ mg/ml. Recovery studies were carried out by adding commercially obtained 5-hydroxytryptamine to aliquots of the various urine samples and processing them as described for the test samples. Recovery for total 5-hydroxyindoles ranged from 99.5% to 119%.

4.8 Results

The results are presented as the mean values obtained from ten rats for each dietary group ± standard error of the mean (S.E.M.).

4.8.1 Statistics

The Student's t-test was used to compare the means of each test group to the control group and the significance was determined at $p<0.05$. 163
4.8.2 Weight Changes

The bracken-fed rats had a slower rate of weight gain compared to the control, shikimate- and quercetin-fed rats (p<0.05) using the Student's t-test (Figure 4.3).

4.8.3 Clinical Signs

There were no overt signs of illness in any of the experimental groups except as described above for the bracken-fed rats.

4.8.4 Urinary pH

The urine was basic in all the groups during the experimental period (Table 4.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (weeks)</th>
<th>4</th>
<th>8</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>8±0</td>
<td>8±0</td>
<td>8±2</td>
</tr>
<tr>
<td>Quercetin</td>
<td></td>
<td>9±0</td>
<td>8±1</td>
<td>9±0</td>
</tr>
<tr>
<td>Bracken</td>
<td></td>
<td>8±0</td>
<td>8±1</td>
<td>8±0</td>
</tr>
<tr>
<td>Shikimate</td>
<td></td>
<td>8±0</td>
<td>8±1</td>
<td>8±1</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
Weights are the means of ten rats per group.

(4) The bracken-fed rats were statistically significantly different from the control group, $p < 0.001$ throughout the study period.
4.8.5 Urinary Nitrite

At week 4, urinary nitrite levels in the quercetin- and bracken fed rats were higher than the control, while the shikimate-fed rats had a lower level. These levels dropped dramatically in the bracken-fed rats, steadily in the quercetin-fed rats and remained constant in the shikimate-fed rats.

In the three test groups there was a decrease in urinary nitrite with time, but in the control group it rose at week 8 and dropped at week 13 (Table 4.2).

### Table 4.2 Urinary Nitrite Concentration in Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>4</th>
<th>8</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.5±0.0</td>
<td>1.0±0.4</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.0±0.2*</td>
<td>1.7±0.3</td>
<td>0.2±0.0^</td>
</tr>
<tr>
<td>Bracken</td>
<td>14.0±0.3*</td>
<td>2.3±0.0*</td>
<td>0.1±0.0^</td>
</tr>
<tr>
<td>Shikimate</td>
<td>0.1±0.0^</td>
<td>0.1±0.0^</td>
<td>0.1±0.0^</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
* = values statistically higher than control (p<0.05)
+ = values statistically lower than control (p<0.05)
(Using the Student's t-test)
4.8.6 Urinary Thiocyanate

The urinary thiocyanate levels for the quercetin-fed rats were higher than the control values throughout the experimental period. In the bracken-fed rats, it was lower at week 8 and higher at week 13. In the shikimate-fed rats it was higher at week 4, lower at week 8 and higher at week 13 (Table 4.3).

Table 4.3 Urinary Thiocyanate Concentration of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>4</th>
<th>8</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.2±0.0</td>
<td>1.8±0.0</td>
<td>12.2±0.3</td>
</tr>
<tr>
<td>Quercetin</td>
<td>13.5±1.0*</td>
<td>5.2±0.1*</td>
<td>21.7±0.7*</td>
</tr>
<tr>
<td>Bracken</td>
<td>2.1±0.0</td>
<td>1.4±0.0+</td>
<td>20.4±0.3*</td>
</tr>
<tr>
<td>Shikimate</td>
<td>5.5±0.1*</td>
<td>2.4±0.1*</td>
<td>14.4±0.4*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
* = values statistically higher than control (p<0.05)
+ = values statistically lower than control (p<0.05)
(Using the Student’s t-test)
4.8.7 Serum Thiocyanate

The bracken-fed rats had a significantly higher level of serum thiocyanate than the control group while the shikimate-fed rats had a significantly lower level than the control group (Table 4.4).

Table 4.4 Serum Thiocyanate Concentration at week 13 in Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Thiocyanate (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.0±0.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5.0±0.3</td>
</tr>
<tr>
<td>Bracken</td>
<td>6.9±0.2*</td>
</tr>
<tr>
<td>Shikimate</td>
<td>2.5±0.2+</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
* = values statistically higher than control (p<0.05)
+ = values statistically lower than control (p<0.05)
(Using the Student’s t-test)

4.8.8 Determination of N-Nitroso Compounds in the Urine and Gut Contents

The determination of N-nitroso compounds in the urine and gut yielded negative results. N-nitroso compounds were not found in the gut contents or urine of any of the experimental groups.
4.8.9 The Nitrosation Assay Procedure on the Gut Contents

Consequent upon the NAP, the intestinal contents of the quercetin-fed rats showed the greatest susceptibility to N-nitrosation followed successively in decreasing order by the stomach contents of the bracken-fed rats, the intestinal contents of the shikimate-fed rats, the caecal contents of the bracken-fed rats, the intestinal contents of the bracken-fed rats, the caecal contents of the quercetin-fed rats and the caecal contents of the shikimate-fed rats. In regional terms, the intestinal contents were most readily susceptible to N-nitrosation. The stomach contents of the quercetin- and shikimate-fed rats were not susceptible to N-nitrosation, nor were the stomach, intestinal and caecal contents of the control group (Table 4.5).

Table 4.5 N-Nitrosatable Compounds in the Gut at week 13 of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets post Nitrosation Assay Procedure.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Stomach Contents</th>
<th>Intestinal Contents</th>
<th>Caecal Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Quercetin</td>
<td>N.D.</td>
<td>345±46*</td>
<td>7±1*</td>
</tr>
<tr>
<td>Bracken</td>
<td>160±15*</td>
<td>61±3*</td>
<td>78±25*</td>
</tr>
<tr>
<td>Shikimate</td>
<td>N.D.</td>
<td>79±4*</td>
<td>4±1*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.

* = values statistically higher than control (p<0.05)

(Using the Student’s t-test)

N.D. = Not Detected
4.8.10 Nitrosation Assay Procedure on the Urine

The urine of the shikimate-fed rats was not susceptible to nitrosation, while the urine of quercetin-fed rats at weeks 4 and 8, and the bracken-fed rats at week 4, were significantly more susceptible to nitrosation than the control group. At week 13 however, the three test groups were less susceptible to nitrosation than the control group (Table 4.6).

Table 4.6 N-Nitrosatable Compounds in the Urine at week 13 of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets post Nitrosation Assay Procedure.

| N-Nitrosatable Compounds (ug NO₂⁻/mg creatinine) |
|------------------|---------------|---------------|
| **Groups**        | **Time (weeks)** |
| Control           | 4             | 8             | 13            |
| Quercetin         | 2.70±0.20*    | 3.00±0.06*    | 0.10±0.02+    |
| Bracken           | 1.20±0.02*    | N.D.+         | 0.70±0.01+    |
| Shikimate         | N.D.          | N.D.+         | N.D.+         |

Values are the means ± S.E.M. of ten rats.
* = values statistically higher than control (p<0.05)
+ = values statistically lower than control (p<0.05)
(Using the Student’s t-test)
### 4.8.11 Urinary Xanthurenic Acid

The three test groups had lower xanthurenic acid levels at week 4, the shikimate group a higher level at week 8, while the quercetin and shikimate groups had lower levels at week 13 (Table 4.7).

#### Table 4.7 Urinary Xanthurenic Acid Concentration of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Xanthurenic Acid (ug/mg creatinine)</th>
<th>Groups</th>
<th>4</th>
<th>8</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>2.60±0.47</td>
<td>0.35±0.06</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td></td>
<td>0.33±0.02*</td>
<td>0.35±0.07</td>
<td>0.11±0.01*</td>
</tr>
<tr>
<td>Bracken</td>
<td></td>
<td>1.00±0.13*</td>
<td>0.26±0.03</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>Shikimate</td>
<td></td>
<td>0.75±0.07*</td>
<td>0.73±0.17*</td>
<td>0.16±0.01*</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.

* = values statistically higher than control (p<0.05)
+ = values statistically lower than control (p<0.05)

(Using the Student’s t-test)
4.8.12 Urinary Kynurenic Acid

The three test groups had lower kynurenic acid levels at week 4 than the control group, while at week 8, the quercetin and bracken groups had lower kynurenic acid levels. At week 13, there were no differences between the test and control groups (Table 4.8).

Table 4.8 Urinary Kynurenic Acid Concentration of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Control</th>
<th>Quercetin</th>
<th>Bracken</th>
<th>Shikimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.92±0.02</td>
<td>0.13±0.01+</td>
<td>0.30±0.01+</td>
<td>0.22±0.01+</td>
</tr>
<tr>
<td>8</td>
<td>0.27±0.04</td>
<td>0.14±0.01+</td>
<td>0.13±0.01+</td>
<td>0.39±0.10</td>
</tr>
<tr>
<td>13</td>
<td>0.07±0.01</td>
<td>0.08±0.01</td>
<td>0.07±0.00</td>
<td>0.06±0.00</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
+ = values statistically lower than control (p<0.05)
(Using the Student's t-test)
4.8.13 Urinary Total 5-Hydroxyindoles

The shikimate-fed rats had a significantly lower level of total 5-hydroxyindoles at week 4 but otherwise there were no significant differences (Table 4.9).

Table 4.9 Urinary Total 5-Hydroxyindoles of Rats fed 1% (w/w) Quercetin, 10% (w/w) Bracken, 1% (w/w) Shikimate and Control Diets.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>4</th>
<th>8</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75±25</td>
<td>35±12</td>
<td>23±7</td>
</tr>
<tr>
<td>Quercetin</td>
<td>68±23</td>
<td>71±24</td>
<td>34±11</td>
</tr>
<tr>
<td>Bracken</td>
<td>45±15</td>
<td>20±7</td>
<td>38±13</td>
</tr>
<tr>
<td>Shikimate</td>
<td>21±5+</td>
<td>47±16</td>
<td>30±10</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.
+ = values statistically lower than control (p<0.05)
(Statistical analysis based on comparison of test values to control for each week, using the Student's t-test)
4.8.14 Urinary Creatinine Values

These were determined for each urine sample and metabolites determined in the urine were expressed per mg creatinine to take into account variations due to volume of urine output and dilution factors. These are presented in Table 4.10.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>4</th>
<th>8</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8±0.1</td>
<td>1.4±0.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.6±0.2</td>
<td>1.5±0.1</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Bracken</td>
<td>1.5±0.1</td>
<td>2.5±0.2</td>
<td>2.6±0.2</td>
</tr>
<tr>
<td>Shikimate</td>
<td>2.1±0.1</td>
<td>1.1±0.1</td>
<td>2.1±0.1</td>
</tr>
</tbody>
</table>

Values are the means ± S.E.M. of ten rats.

4.9 Discussion

The hypothesis tested by these studies was that N-nitrosation was a possible mechanism for the mediation of bracken-induced carcinogenicity.

To this end the urinary nitrite levels were monitored over a 90-day experimental period by measuring at three time points during the experimental period. The progressive decline of nitrite levels in the urine of the quercetin- and bracken-fed rats may be suggestive of the utilisation of nitrite for the formation of N-nitroso compounds, but may also indicate impairment of the nitrate reductase responsible for the reduction of nitrate to nitrite. Cyanide which may be produced upon enzymatic hydrolysis of cyanogenic glycosides, is a potent inhibitor of nitrate reductase (Solomonson, 1981). The activity of the enzyme is enhanced by hydrogen donors such as malate, succinate and glutamate. Another mechanism that might
impair its activity in bracken-intoxicated animals is via the anti-thiamine effect which deprives the animal of an essential co-factor (thiamine pyrophosphate) for the enzymes pyruvate dehydrogenase and α-glutamate dehydrogenase responsible for the metabolism of these hydrogen donors via the citric acid cycle.

The determination of the levels of thiocyanate in the urine at three time points over the experimental period and in the serum at the end of the experiment would suggest that the conditions for in vivo N-nitrosation exist in the three experimental groups. However, no N-nitroso compounds were detected until the application of the Nitrosation Assay Procedure when some N-nitrosatable material was detected in the test groups.

Some explanations which may be advanced for this are, the insufficient sensitivity of the colorimetric method of Eisenbrand and Preussmann (1970). Although this method is described as sensitive, it cannot be compared to the newer methods based on selective gas-liquid chromatography and linked to mass spectrometers (Fiddler, 1975; Webb et al., 1979). The other possible explanation could be the role of the blocking agents like caffeic and ferulic acids which occur in bracken and could block N-nitrosation in the bracken-fed rats. Thus a study of the kinetics of the process of N-nitrosation in the bracken-fed rats may help to delineate the relative roles of the factors that favour N-nitrosation and balance them against those that block N-nitrosation.

The situation with quercetin and shikimate is interesting in view of the nitrosatability of the gut contents of rats fed these bracken constituents, as shown by the NAP. It may be explained on the basis of their ability to alter the composition of the gut microflora in such a way that the growth of bacteria capable of enhancing N-nitrosation is preferentially promoted in the gut of animals fed these compounds (Sander, 1968). The ability of quercetin to act as a catalyst of N-nitrosation (Pignatelli et al., 1980) must be a further important factor in the very high levels of N-nitrosatable compounds found in the intestine of the quercetin-fed rats after the NAP. In addition the urinary levels of thiocyanate, a potent catalyst of N-nitrosation, in the quercetin-fed rats, rises very rapidly at week 4 and must be another important factor responsible for the susceptibility of the intestinal contents of the rats to N-nitrosation. This initially very high levels of thiocyanate is probably

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due to the enhanced activity of rhodanese (3.10.5). The levels of thiocyanate drops in the quercetin, bracken and shikimate groups at week 8, a phenomenon previously observed in rats on chronic cyanide ingestion and attributed to substrate saturation (Smith and Foulkes, 1966). However, the levels in the control group also drop at week 8 and although the decrease is less remarkable than in the test groups it perhaps represents a natural pattern of thiocyanate excretion in rats reflective of gastro-intestinal recirculation of thiocyanate as the animals increase in size and the amount of space available for thiocyanate recirculation increases, and the amount excreted drops (Kreutler et al., 1978; Okoh, 1978). The thiocyanate levels rise at week 13 and this may be reflective of saturation re-absorption in the renal tubules (Kreutler et al., 1978).

The scenario provided by initial high nitrite and thiocyanate levels is ideal for N-nitrosation to occur. It may be concluded therefore that this occurs early in the course of feeding the test compounds. Indeed N-nitrosatable compounds were detected in the urine as early as week 4 (Table 4.6).

The detection of N-nitrosatable compounds in the stomach of the bracken-fed rats only, but not in the quercetin- and shikimate-fed rats may be attributed to two factors, namely, the absence of any nitrosatable compounds (e.g. amines or amides) in the stomach of the quercetin- and shikimate-fed rats and the absence of bacteria capable of promoting the process of nitrosation in the stomach. Thus although there is nitrite and the catalyst thiocyanate is available and the acidic pH of the stomach is ideal for nitrosation, the absence of an amine or amide makes it impossible for the process to take place in the stomach of these rats. Bracken on the other hand contains a primary amide, pterolactam and upon ingestion of bracken, this compound is readily available in the stomach to be nitrosated.

In contrast, the intestinal and caecal contents of the three test groups are susceptible to N-nitrosation due to the presence of the nitrosatable compounds produced in part as a result of bacterial metabolism as well as other factors such as the presence of thiocyanate and in the case of bracken, the potentially nitrosatable compound, pterolactam. It is possible then to envisage a balance of factors in each of the three test groups which promote or inhibit N-nitrosation. In the three test groups there are common factors like the presence of nitrite and thiocyanate.
However, while quercetin is able to produce more thiocyanate than the others it lacks the presence of N-nitrosatable compounds, but may produce these via the metabolism of gut flora. On the other hand, while bracken contains the potentially nitrosatable compound pterolactam, it also contains caffeic acid and ferulic acid, both blockers of N-nitrosation. In the shikimate-fed rats, it is probably the metabolism of gut flora that is most important with regards to the production of N-nitrosatable compounds.

The possible role of tryptophan metabolism in bracken-induced carcinogenesis was investigated to determine if any of the metabolites might be considered significant in the process.

The lower levels of xanthurenic and kynurenic acids in the bracken-fed rats are in agreement with the results of Dalgleish (1955) who reported decreased urinary excretion of the metabolites of the kynurenine pathway in thiamine deficient rats.

The quercetin- and shikimate-fed rats also had lower levels of both metabolites in the urine compared to the control group except for the shikimate-fed group at week 8. It is thus pertinent to consider whether the effect is related to or mediated via an anti-thiamine mechanism. It had been suggested that some thermostable anti-thiamine factors such as isoquercitrin and caffeoyl-shikimic acid are only active in vitro. The activity of the thermostable anti-thiamine factor, "the SF factor" (Konishi, 1984) in vivo in the equine and the observation here, of the lowered kynurenic and xanthurenic levels in the quercetin- and shikimate-fed rats appear to be indicative of possible in vivo anti-thiamine activity of constituents of bracken other than thiaminase.

It is pertinent to note that the xanthurenic and kynurenic acid levels were most significantly lower in the test groups at week 4 which is also the period during which the anti-thiamine activity of bracken is most prominent, when ataxia was evident. Is this suggestive of in vivo anti-thiamine activity by shikimate and quercetin?

This does not rule out the possibility of a direct inhibitory effect of the test compounds on any or several of the enzymes responsible for the metabolism of tryptophan to produce kynurenic and xanthurenic acid (Figure 4.2). In addition it ought to be borne in mind that tryptophan metabolism is
influenced by the nature of the gut flora (Williams, 1972). This is in turn influenced by the diet and therefore any combination of factors including an action via the antithiamine effect indirectly influencing the enzymes themselves or an alteration in the gut flora component of tryptophan metabolism may serve to explain the differences in tryptophan metabolism in the three test groups compared to the control.

However the examination of the pathway of tryptophan metabolism by examining the level of total 5-hydroxyindoles in the urine did not reveal any significant differences between the test and control groups except for the shikimate group at week 4. It would be expected that these metabolites may be excreted in larger quantities in the thiamine-deficient animals where the kynurenine pathway is impaired.

An hypothesis to explain this apparent lack of shift towards indole pathway may be that the 5-hydroxyindoles so produced have been N-nitrosated by the nitrite which was found to attain high levels in quercetin- and bracken-fed rats at week 4, hence the apparent absence of elevated 5-hydroxyindoles in the test groups, which would normally be expected. When rat peritoneal mast cells were treated in vitro with some fractions of bracken, elevated levels of histamine were detected. Two β-glucopyranosides called braxin A1 and A2 were identified as responsible for this activity of bracken (Saito and Mochizuki, 1986). It is therefore plausible to suggest that the shift towards the indole pathway may be undetectable in vivo due to the concomitant and overriding effect of N-nitrosation.

Therefore it is not certain whether the metabolism of tryptophan is significant in bracken-induced carcinogenesis, especially as the specific identity of the N-nitrosatable compounds produced in the NAP on bracken-, quercetin- and shikimate-fed animals is unknown. If the identity of these N-nitrosatable compounds is known, it may be possible to affirm the view that there is a shift away from the kynurenine pathway towards the indole pathway and that the products of that pathway are involved in N-nitrosation.
4.10 Conclusion

The potential of bracken-fed rats for N-nitrosation has been demonstrated by these studies, but the identity of the bracken carcinogen is still unknown.

The role of tryptophan metabolism in the carcinogenicity of bracken was not conclusive from these studies but the lower levels of xanthurenic and kynurenic acids in the test rats is consistent with the pattern in thiamine-deficient animals (Dalgleish, 1955). The significance of thiamine deficiency in relation to the process of N-nitrosation and tryptophan metabolism may be more apparent if it is considered that mice on a vitamin B complex-deficient diet and treated with arecoline and potassium nitrite had a higher incidence of tumours than mice on a normal diet (Bhide et al., 1984). While it is possible to attribute these effects to other constituents of the B-complex group of vitamins other than thiamine, it is nevertheless an important point to consider the possible role of the anti-thiamine phenomenon as a contributor to the enhancement of N-nitrosation, and the alteration of tryptophan metabolism. The hypothesis being put forward here is that the products of altered tryptophan metabolism in thiamine-deficient animals may be available for N-nitrosation.
CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION
General Discussion

The central issue that remains unresolved in studies on bracken carcinogenesis is the identity of the carcinogen(s), and it is hoped that from it will proceed an understanding of the pathogenesis of the disease, and the mechanisms of action whereby cancer is induced in bracken-intoxicated animals. The identification of the bracken carcinogen may reveal a new class of compounds hitherto unrecognised, or a familiar class of compounds, hitherto thought to be safe. This area of study thus has immense scientific and public health importance.

Various approaches have been attempted in trying to determine the identity of the bracken carcinogen(s) but none of the candidate carcinogens nominated so far has met all the requirements nor explained the various features of bracken-induced carcinogenesis.

In Chapter Two, an initiation-promotion hypothesis was examined as an explanation for the mediation of bracken-induced carcinogenesis. The ability of shikimate on its own to enhance ornithine decarboxylase in the tissues of rats so treated suggests a tumour promoting ability. This implies the presence of a tumour initiator. It has not been possible to determine what this might be, but some candidates can be suggested including quecetin and ptaquiloside, both of which are mutagens.

From the studies in Chapter Four, it was concluded that the nitrosation of some nitrosatable entities in bracken may offer a basis for explaining bracken-induced carcinogenesis. It was possible to identify one such potentially nitrosatable compound as pterolactam, but it is probably not the only one.

The nitrosatable compounds formed in bracken-intoxicated animals would probably be nitrosamides rather than nitrosamines, although a mixture of both cannot be discounted. The nitrosamides tend to attack organs of rapid cell turnover such as the intestinal tract and the bone marrow, that is they tend to act in a "radiomimetic" manner. The nitrosamines tend for the most part to be organ-specific. Where specific organs like the liver are attacked, the nitrosamides tend to induce periportal rather than centriloculular necrosis which is seen in toxic injury induced by nitrosamines. The lesions observed in the liver of the bracken-fed rats in
the 28-day study (Chapter Three) were those of periportal necrosis. The bracken-induced toxicity in bovines consists of the shorter term bovine bracken poisoning caused by bone marrow aplasia with resultant haemorrhaging and the longer term alimentary tract and bladder tumours. Both these lesions could be attributable to the actions of compounds acting in a radiomimetic manner and the nitrosamides serve a unifying hypothesis for these syndromes. Apart from the sub-acute and chronic lesions which may be explained by the actions of a nitrosamide, the teratogenic effects of bracken may also be explained on that basis. For while the nitrosamides are capable of teratogenicity, the nitrosamines are generally incapable of this except where infused intravenously late in pregnancy (Magee et al., 1976). Bracken has been amply demonstrated to be capable of teratogenicity (Yasuda et al., 1979). The nitrosamides are also powerful mutagens being active in all the usual microbial test systems and in Drosophila, while the nitrosamines are active in Drosophila, but require activation to be active in the microbial assay systems (Magee et al., 1976). This is due to the requirement of nitrosamines for activation while the nitrosamides do not require activation but tend to decompose to active species (Lobl, 1972). This may also account for the failure in this and several other previous studies to identify a bracken carcinogen intact, because of the ease and rapidity of decomposition of the nitrosamides.

From the foregoing, the unifying role of the "nitrosamide hypothesis" in serving as a basis for the biological activity of bracken is evident, but it need not be the only explanation for the various effects observed in intoxicated animals, for as has been shown in Chapter Two, shikimate is a tumour promoter by virtue of its ornithine decarboxylase enhancing property. Besides it was also shown to be capable of inducing bone marrow aplasia. It is debatable to what extent and degree, shikimate can influence these changes. That shikimate, a compound widely distributed in various human foods should bring about these actions is of public health significance, but it ought to be borne in mind that in the mice, shikimate had induced both gastric carcinoma and leukaemia (Evans and Osman, 1974). It is not unusual for rats to be more resistant to some chemical compounds to which mice are more susceptible (Evans et al., 1982). The tumour promoting activity of shikimate may therefore exist in parallel with a full carcinogenic effect due to another compound, which as suggested above might be a nitrosamide.
The role of quercetin is less clear. It was able to induce bone marrow hypoplasia in the rat as seen in Chapter Two but its carcinogenic properties were not so clearly defined. However quercetin is a mutagen, a catalyst of N-nitrosation and in one report, a carcinogen. It is possible to speculate that the one report of its carcinogenicity (Pamukcu et al., 1980b) was due to the presence of nitrosatable entities as contaminants in the feed and the presence of quercetin thus catalysed a process which would in its absence probably be reversed by normal detoxification mechanisms. Animal feed may occasionally be contaminated by nitrosamines and nitrosatable entities (Silverman and Adams, 1983). It would appear that this one report by Pamukcu et al. (1980b) is an aberrant report complicated by extenuating factors, one of which has been speculated upon above. It remains however to determine if quercetin might be an initiator. It is a line of inquiry worth pursuing if possible by combining it with shikimate in an initiation-promotion assay. That quercetin may act as a promoter in conjunction with other chemicals is illustrated by the results obtained for ornithine decarboxylase when quercetin was combined with cyclophosphamide (Tables 2.4 and 2.7), saccharin (Table 2.5), lithocholate (Table 2.6), BBN (Table 2.8) and MNNG (Table 2.9). These further buttress the need for a future line of inquiry to re-examine quercetin’s role in bracken-induced carcinogenesis especially its interaction with other constituents of bracken.

The acute or sub-acute toxic effects of bracken include the avitaminosis B1 which in ruminants causes cerebrcortical necrosis and in monogastrics causes ataxia. As was investigated in Chapter Three, the latter may be caused in part by the cyanogenic glycoside prunasin, which upon hydrolysis yields cyanide, and nitrite, which was found in very high levels in the urine of bracken-fed rats. Nitrite so detected may arise from the reduction of nitrate which is very commonly found in vegetables and plants, or may arise as a breakdown product of N-nitroso compounds. In addition, the gut flora may produce nitrite as part of its metabolic profile. Any of these three possibilities may occur with the bracken-intoxicated animals and is clearly deserving of further investigation. But here again, a case could be made for the role of the nitrite from the breakdown of the nitrosamide as a co-contributor to the development of ataxic neuropathy in bracken-intoxicated monogastrics. The unifying nature of the nitrosamide hypothesis thus spans the acute syndromes both in monogastrics and ruminants and the chronic (carcinogenic) syndrome in all species tested so far. In any case however, it is unlikely that there will be one single agent directly
responsible alone for all the signs seen in bracken poisoning. Bracken toxicity remains a multifactorial phenomenon and much remains to be done to identify the role of each of these factors in the overall context of bracken toxicity.

In summary the main conclusions of this study are:

1. Shikimate is a tumour promoter based on the enhanced activity of ornithine decarboxylase in the tissues of rats fed shikimate. It is also capable of inducing bone marrow aplasia in rats.

2. Cyanide and nitrite are additional contributory factors to the development of ataxia in bracken-intoxicated rats.

3. N-nitrosation was identified as a potentially significant process in the mediation of bracken-induced carcinogenesis, and it was suggested that though the identity of any nitroso compounds was not determined, the characteristics of bracken-induced carcinogenesis, suggested that nitrosamide as the likely cause.

4. Although quercetin was shown to be capable of inducing bone marrow aplasia, its initiating, promoting or carcinogenic role could not be conclusively determined. Quercetin, however, is a catalyst of N-nitrosation which suggests that quercetin may play a more critical role in bracken-induced carcinogenesis than hitherto thought.
APPENDIX
# Non-Carcinogenic Effects of Bracken in Livestock and Laboratory Animals

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose of Bracken in the Diet</th>
<th>Clinico-Pathologic Signs</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bovine</strong></td>
<td>Not stated (field cases)</td>
<td></td>
<td>Storrar (1893) Penberthy (1893) Almond (1894)</td>
</tr>
<tr>
<td></td>
<td>Not stated (field cases)</td>
<td>Hyperpyrexia (106°F-108°F) Anorexia, dyspnoea Petechial and ecchymotic haemorrhages.</td>
<td>Evans and Evans (1949)</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>Thrombocytopenia Leucopenia</td>
<td>Naftalin and Cushnie (1951, 1956) Heath and Wood (1958)</td>
</tr>
<tr>
<td></td>
<td>40-70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7lb/day for 23 days</td>
<td>Dyspnoea ataxia</td>
<td>Hawden and Bruce (1917)</td>
</tr>
<tr>
<td><strong>Equine</strong></td>
<td>25-50%</td>
<td>Anorexia, ataxia avitaminosis B₁, elevated pyruvate eosinopenia and thrombocytopenia</td>
<td>Evans et al. (1951)</td>
</tr>
<tr>
<td></td>
<td>33.29 kg/66 days</td>
<td>Hyperpyrexia (102°F-108°F) Generalised haemorrhaging</td>
<td>Moon and Raafat (1951a)</td>
</tr>
<tr>
<td><strong>Ovine</strong></td>
<td>Not stated (field cases)</td>
<td>Leucopenia, thrombocytopenia</td>
<td>Parker and McCrea (1965)</td>
</tr>
<tr>
<td>Species</td>
<td>Dose of Bracken in the Diet</td>
<td>Clinico-Pathologic Signs</td>
<td>References</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------</td>
<td>------------------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Ovine</td>
<td>Not stated (field cases)</td>
<td>Retinal degeneration (&quot;Bright blindness&quot;)</td>
<td>Watson et al. (1965)</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>Thrombocytopenia and leucopenia</td>
<td>Watson et al. (1972)</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>Reduced serum lactate dehydrogenase activity</td>
<td>Watson et al. (1972)</td>
</tr>
<tr>
<td></td>
<td>15-33%</td>
<td>Ataxia</td>
<td>Evans et al. (1975)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cerebro-cortical necrosis</td>
<td></td>
</tr>
<tr>
<td>Porcine</td>
<td>25-33%</td>
<td>Unthriftiness, avitaminosis B&lt;sub&gt;1&lt;/sub&gt;, elevated pyruvate. Reduction of reticulocyte count</td>
<td>Evans et al. (1963)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Harding (1972)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Evans et al. (1972)</td>
</tr>
<tr>
<td>Hooded Wistar Rats</td>
<td>40%</td>
<td>Cachexia, avitaminosis B&lt;sub&gt;1&lt;/sub&gt;, anorexia, polyneuritis and ataxia</td>
<td>Evans and Evans (1949)</td>
</tr>
<tr>
<td>Black Rats</td>
<td>50%</td>
<td>Cachexia, avitaminosis B&lt;sub&gt;1&lt;/sub&gt;, alopecia, anorexia, thrombocytopenia, leucopenia, atrophy of spleen and myocardium, bone marrow aplasia.</td>
<td>Cordy (1952)</td>
</tr>
<tr>
<td>Species</td>
<td>Dose of Bracken in the Diet</td>
<td>Clinico-Pathologic Signs</td>
<td>References</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------</td>
<td>-----------------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Sprague Dawley Rats</td>
<td>50%</td>
<td>Leucopenia, thrombocytopenia, neutrophilia, erythropenia, anorexia, cachexia and depression</td>
<td>Schacham et al. (1970)</td>
</tr>
<tr>
<td>Guinea Pigs</td>
<td>ad libitum</td>
<td>Haemorrhagic bladders</td>
<td>Evans et al. (1967)</td>
</tr>
<tr>
<td>Rabbits</td>
<td>ad libitum</td>
<td>Haemorrhagic bladders</td>
<td>Evans et al. (1967)</td>
</tr>
<tr>
<td>ICR-JCL Mice</td>
<td>33%</td>
<td>Intrauterine growth suppression in pregnant mice, rib variations and retarded sternebral ossification</td>
<td>Yasuda et al. (1974)</td>
</tr>
<tr>
<td>C57Bl/6 Mice</td>
<td>33%</td>
<td>Increased abortions</td>
<td>Fushimi et al. (1973)</td>
</tr>
</tbody>
</table>
## Appendix II

**The Carcinogenic Effects of Bracken in Livestock and Laboratory Animals**

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of Tumour</th>
<th>Location</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Adenocarcinoma</td>
<td>Ileum</td>
<td>Evans and Mason (1965)</td>
</tr>
<tr>
<td></td>
<td>Adenoma</td>
<td></td>
<td>Sumi et al. (1981)</td>
</tr>
<tr>
<td></td>
<td>Sarcoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Carcinoma</td>
<td></td>
<td>Pamukcu and Price (1969)</td>
</tr>
<tr>
<td></td>
<td>Papilloma</td>
<td>Urinary bladder</td>
<td>Hirono et al. (1970)</td>
</tr>
<tr>
<td>Mice</td>
<td>Adenoma</td>
<td>Lung and stomach</td>
<td>Evans (1968)</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytic leukaemia</td>
<td>Bone marrow</td>
<td>Evans (1976)</td>
</tr>
<tr>
<td></td>
<td>Carcinoma</td>
<td>Fore stomach</td>
<td>Pamukcu et al. (1972)</td>
</tr>
<tr>
<td></td>
<td>Papilloma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japanese quail</td>
<td>Adenocarcinoma</td>
<td>Ileum, caecum and colon</td>
<td>Evans et al. (1967)</td>
</tr>
<tr>
<td>Hamster</td>
<td>Adenocarcinoma</td>
<td>Ileum and caecum</td>
<td>Evans (1968)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>Papillary carcinoma and adenocarcinoma, transitional cell carcinoma</td>
<td>Bladder</td>
<td>Evans et al. (1967)</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>Jejunum</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Type of Tumour</td>
<td>Location</td>
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</tr>
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<td>-------------</td>
<td>---------------------------------</td>
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<td>-----------------------------</td>
</tr>
<tr>
<td>Egyptian toad</td>
<td>Adenocarcinoma</td>
<td>Ileum</td>
<td>El-Mofty et al. (1980)</td>
</tr>
<tr>
<td></td>
<td>Hepatoma</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>Haemangioma and papilloma</td>
<td>Urinary bladder</td>
<td>Rosenberger and Heeschen (1960)</td>
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<td></td>
<td>Adenoma and adenocarcinoma</td>
<td>Intestines</td>
<td>Sofrenovic et al. (1965)</td>
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<td></td>
<td>Squamous carcinoma and papilloma</td>
<td>Oesophagus</td>
<td>Jarrett et al. (1978)</td>
</tr>
<tr>
<td>Ovine</td>
<td>Fibrosarcoma</td>
<td>Maxilla and mandible</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transitional cell carcinoma</td>
<td>Bladder</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leiomyosarcoma</td>
<td></td>
<td>McCrea and Head (1978, 1981)</td>
</tr>
<tr>
<td></td>
<td>Papilloma</td>
<td>Rumen, intestine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>and colon</td>
<td>Evans (1972)</td>
</tr>
<tr>
<td></td>
<td>Carcinoma</td>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphosarcoma</td>
<td>Thymus and kidney</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymoma</td>
<td>Thymus</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX III - Structures of Some Toxicologically Significant Constituents of Bracken

Quercetin

Kaempferol

Isoquercitrin

Rutin

Hesperidin

Naringin
Shikimic acid

Prunasin

Ferulic acid

Caffeic acid

A Tannin (corilagin)

Fumaric acid

Pterolactam
<table>
<thead>
<tr>
<th>Structure</th>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Pterosin Z</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>I</td>
<td>Pterosin I</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>I</td>
<td>Pteroside Z</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>I</td>
<td>(3R) pterosin D</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-OH</td>
</tr>
<tr>
<td>I</td>
<td>(3S) pteroside S</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Glu</td>
<td>-OH</td>
</tr>
<tr>
<td>II</td>
<td>(2S) pterosin A</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>II</td>
<td>(2S) pterosin K</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Cl</td>
<td>-H</td>
</tr>
<tr>
<td>II</td>
<td>(2H, 3R) pterosin L</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-OH</td>
</tr>
<tr>
<td>II</td>
<td>(2S) pteroside A</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Glu</td>
<td>-H</td>
</tr>
<tr>
<td>II</td>
<td>(2R) pterosin B</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>II</td>
<td>(2R) pterosin F</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Cl</td>
<td>-H</td>
</tr>
<tr>
<td>II</td>
<td>(2R) pterosin E</td>
<td>-COOH</td>
<td>-H</td>
</tr>
<tr>
<td>III</td>
<td>(2S) pterosin D</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>III</td>
<td>(2R) palmityl pterosin B</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Glu</td>
<td>-H</td>
</tr>
<tr>
<td>III</td>
<td>Acetyl-δ&lt;sup&gt;-&lt;/sup&gt;-dehydropterolin B</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Glu</td>
<td>-H</td>
</tr>
<tr>
<td>III</td>
<td>(2R) pteroside B</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-OH</td>
</tr>
<tr>
<td>III</td>
<td>(2S, 3S) pterosin C</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-OH</td>
</tr>
<tr>
<td>III</td>
<td>(2S, 3S) pterosin J</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-OH</td>
</tr>
<tr>
<td>III</td>
<td>(2S, 3S) acetylpterosin C</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Glu</td>
<td>-OH</td>
</tr>
<tr>
<td>IV</td>
<td>(25) pterosin G</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>V</td>
<td>Pterosin N</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>V</td>
<td>(2S) pterosin P</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>-H</td>
</tr>
<tr>
<td>V</td>
<td>(2S) pteroside P</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;Glu</td>
<td>-H</td>
</tr>
</tbody>
</table>
Aquilide A (Ptaquiloside)

Glucose

Pterosin B

Activated compound

Acid conditions

Alkaline conditions
## Appendix IV

### Average Calculated Composition of Normal Rodent Diet

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>8.8</td>
</tr>
<tr>
<td>Ether extract (%)</td>
<td>3.5</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>21.5</td>
</tr>
<tr>
<td>Crude fibre (%)</td>
<td>2.7</td>
</tr>
<tr>
<td>Gross energy (MJ/kg)</td>
<td>17.2</td>
</tr>
<tr>
<td>Digestible energy (MJ/kg)</td>
<td>14.2</td>
</tr>
<tr>
<td>Lysine (%)</td>
<td>1.1</td>
</tr>
<tr>
<td>Methionine (%)</td>
<td>0.39</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.90</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>0.80</td>
</tr>
<tr>
<td>Vitamin A (i.u./kg)</td>
<td>11,000</td>
</tr>
<tr>
<td>Vitamin D (i.u./kg)</td>
<td>1,200</td>
</tr>
<tr>
<td>α-tocopherol (i.u./kg)</td>
<td>24</td>
</tr>
<tr>
<td>Vitamin K (mg/kg)</td>
<td>10</td>
</tr>
<tr>
<td>Riboflavin (mg/kg)</td>
<td>7</td>
</tr>
<tr>
<td>Pyridoxine (mg/kg) added</td>
<td>1</td>
</tr>
<tr>
<td>Panthothenic acid (mg/kg)</td>
<td>17</td>
</tr>
<tr>
<td>Nicotinic acid (mg/kg)</td>
<td>80</td>
</tr>
<tr>
<td>Choline chloride (mg/kg)</td>
<td>450</td>
</tr>
<tr>
<td>Cyanocobalamin (mcg/kg)</td>
<td>15</td>
</tr>
<tr>
<td>Manganese (mg/kg)</td>
<td>65</td>
</tr>
<tr>
<td>Iron (mg/kg)</td>
<td>100</td>
</tr>
<tr>
<td>Iodine (mg/kg)</td>
<td>0.5</td>
</tr>
<tr>
<td>Copper (mg/kg)</td>
<td>20</td>
</tr>
<tr>
<td>Zinc (mg/kg)</td>
<td>40</td>
</tr>
<tr>
<td>Cobalt (mg/kg)</td>
<td>1</td>
</tr>
</tbody>
</table>
REFERENCES


EVANS, W.C., EVANS, I.A., HUMPHREYS, D.J., LEWIN, B., DAVIES, W.E.J. and
AXFORD, R.F.E. (1975) Induction of thiamine deficiency in sheep with
lesions similar to those of cerebro-cortical necrosis. J. Comp. Path., 85:
253-267.

EVANS, W.C., JONES, N.R. and EVANS, R.A. (1950) The mechanism of the anti-
aneurin activity of bracken (Pteris aquilina). Biochem. J., 46: XXXVIII-
XXXIX.

EVANS, W.C., WIDDOP, B. and HARDING, J.D.J. (1972) Experimental poisoning


44: 4217-4223.

FARROW, E.P. (1917) On the ecology of the vegetation of Breckland. V.

FERNALD, M.L. and KINSEY, A.C. (1943) Edible Wild Plants of Eastern North


FISCHER, S.M., MILLS, G.D. and SLAGA, T.J. (1982) Inhibition of mouse skin
tumour promotion by several inhibitors of archidonic acid metabolism.
Carcinogenesis, 3: 1243-1245.

FLAVELL, D.J. and LUCAS, S.B. (1982) Potentiation by the human liver fluke,
Opisthorchis viverrini, of the carcinogenic action of N-nitrosodimethylamine

FLAVELL, D.J. and LUCAS, S.B. (1983) Promotion of N-nitrosodimethylamine-
initiated bile duct carcinogenesis in the hamster by the human liver fluke,


FOGGIE, A. (1951) Suspected bracken poisoning in sheep. Vet. Rec., 63:
242.

FORENBACHER, S. (1950) Equisetum poisoning in horses and the vitamin B
Arh., 20: 405-471.

Agric. Fish., Engl. and Wales, p.VI.


205


POTT, E. (1907) Handbuch der tierischen Ernahrung und landwirtschaftlichen Futtermittel. Vol. II.


