DOCTOR OF PHILOSOPHY

# Comparative studies in the pathology of radiomimetic conditions

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*Award date:*
1965

*Awarding institution:*
Bangor University

# COMPARATIVE STUDIES IN THE PATHOLOGY OF RADIOMIMETIC CONDITIONS

A THESIS

Submitted to the University of Wales by

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in candidature for the degree of

Philosophiae Doctor

Bangor, Wales.

September, 1965.

## ACKNOWLEDGEMENTS.

I am indebted to Professor W. Charles Evans and Dr. I. Antice Evans for suggesting the topic, and for their encouragement, assistance and advice during the work.

I would like to thank the following people who have very kindly given help and advice on different aspects of this work.

Dr. R. Schoental and Dr. Magee, M.R.C. Toxicology Unit, Carshalton.

Mr. L.E. Hughes, M.R.C.V.S., Superintending V.I.O., V.I.C., Cambridge.

Mr. W.H. Parker M.R.C.V.S. and Mr. C.T. McCrea M.R.C.V.S., V.I.C., Thirsk, Yorkshire.

Dr. N. Runham, Zoology Dept. U.C.N.W., Bangor.

Mr. Rowlands M.R.C.V.S., Mr. C.M.Edwards M.R.C.V.S. and Mr. T. Parry, Bryn Adda V.I.C., Bangor.

Dr. Scott, Dept. of Pathology, Royal Free Hospital, London.

Mr Edwards, College Farm, Aber.

I wish to acknowledge, with thanks, the help given by the Technical Staff of this Department, in particular Mr. E.C. Hughes, Head Technician.

My thanks are also due to Mr. R.F.E. Axford, M.A., Vet. M.B., M.R.C.V.S., for his help and interest and to Mr. I. Farr for technical assistance.

I am indebted to my wife for her help in the preparation of this thesis and for technical assistance during the course of the work.

I am grateful to the Department of Scientific and Industrial Research for Financial support during the period of the investigations.

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Addition to list of acknowledgements:-

Mr. E. D. Evans, M.R.C.V.S., V.I.C., Liverpool.

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## SUMMARY.

The work was an investigation of the pathology of conditions produced by the toxic factors in the bracken fern.

The parallelism between the syndromes produced by bracken ingestion and the effects of irradiation was considered, and the radio-mimetic nature of the toxic activity was confirmed.

The possible involvement of immunological mechanisms in the pathogenesis of bovine bracken poisoning has been suggested.

The occurrence of generalized Mast Cell increases, especially during the terminal phases has been confirmed, and the releases of heparin and histamine shown to follow some of the Mast Cell elevations. The increases are usually of the order of four-fold and occasionally as much as seven-fold over the normal control level, and can take place within 24-48 hours, which necessitates the daily biopsy sampling of subcutaneous connective tissue during the critical periods. This, in the absence of mitoses in the Mast Cell population, is taken as partial evidence to support the case that the cells are derived from mobile precursors probably of lymphoid origin.

Other cell types have been shown to be involved in the syndrome, in addition to the normal inflammatory cells. "Giant" or multinucleate cells, which are apparently formed by the coalescence of active mononuclear cells, appear at the times of Mast Cell peaks. The "Giant" cells occur particularly when the calves have been presensitized with small amounts of bracken before the main quantity was given. It is suggested that these animals showed manifestations of delayed hypersensitivity.

Plasma cell appearances have been followed and these suggest an important if not fundamental role in the aetiology of the condition.

A mechanism has been put forward to explain the features of the condition i.e. that in some way the bracken toxin causes the formation of new cellular antigens, and a subsequent immunological reaction causes their rejection and the observed capillary damage.

To test this hypothesis calves which had been fed small quantities of bracken were challenged by intra-dermal injection of an ethanolic extract of bracken, in contrast to control calves not having had contact with bracken, they showed reactions of the delayed hypersensitivity type. Further fractionation towards the causative agent using this as an assay was attempted. A fraction was obtained which consisted of 4% by weight of the original ethanol extract. This contained the major part of the allergic activity. Fractions were given *per os* to calves and the results indicate the probable common identity of the allergic factors and the agents responsible for the bovine bone marrow damage.

The action of the bracken is in many ways analogous to irradiation. It was possible that features similar to the more immediate effects and conversely to the more delayed effects could be shown.

Sodium and Potassium balances on the whole animal have shown in three cases of which two were fatal, that serious Sodium losses did not occur. A histological examination of the intestine was made and damage analogous to irradiation effects was shown.

Rats fed with bracken as 30% of the diet, for 64 days, have developed extensive intestinal tumours which

cause fatalities six months later. All the rats given bracke have developed these lesions, indicating the presence of powerful carcinogenic activity.

The tumour induction is of interest because of a possible link between an immunological reaction and tumour production.

# GENERAL INTRODUCTION.

The discovery of the nitrogen and sulphur mustards has led to the synthesis and study of many types of irradiation-simulating compounds.

The natural occurrence in plants of compounds with similar properties has been demonstrated, the Vinblastine family of double indole-indoline alkaloids from the Vinca Rosea Linn. or common periwinkle plant (Johnson et al. 1960 and Noble, Beer and Cutts 1958), while the extraction of Soybean oil meal with Trichloroethylene has been shown to result in the generation of Di-chlorovinyl cysteine which is highly active in the bovine (Schultze et al. 1959) but less so in other species (Pritchard et al. 1956).

The bracken plant (Pteridium aquilinum) has been shown to contain a factor producing a condition known as "Bracken poisoning" in the bovine, which has many of the biological properties of these types of compounds. Evans, Evans and Hughes (1954) and Naftalin and Cushnie (1954) describe the condition as one in which there is bone marrow damage pan-myeloid in nature, pyrexia and often gut lining damage and ulceration and almost invariably widespread petechial haemorrhages.

All these are similar to the changes occurring after large doses of ionizing radiations.

The clinical manifestations of whole body irradiation in mammals varies with the actual dose given and with the time after exposure.

Four characteristic syndromes have been described.

The central nervous system syndrome, produced by massive supralethal amounts of irradiation (12,000r or more) appears promptly and results in death within hours. The syndrome seems to be initiated chiefly by a very great increase in the potassium level of the blood resulting from the failure of the "sodium pump". The cells losing potassium in exchange for the sodium they gain. The condition may also be mediated by a systemic release of histamine. Anticonvulsive drugs such as pentobarbital and diphenyl-hydantoin may delay the onset of convulsions and prolong the survival time as much as twelvefold, but have little effect on the ultimate result (Laird R.D. 1956).

The gastro-intestinal syndrome produced by high, generally supralethal doses of irradiation (1500r) causes death within a period of a few days, primarily the cause is failure of the absorbtive function of the intestine. Epithelial damage of the small intestine prevents sodium excreted as bile salts being reabsorbed and renders the animal prone to bacterial attack at this site and subject to the effects of bacterial products, as well as general toxaemia. Mast cell involvement with heparin, histamine and possibly enzyme release may also be important. Transitory changes in the blood cells, especially neutrophils, platelets and lymphocytes have been observed, related probably to stress effects and to direct cell damage.

The Haemopoietic syndrome is produced by doses that are in the lethal range for most mammals (600r). Deaths usually occur in the second week or later. It is characterized by profound pan-myeloid bone marrow depression resulting in acute thrombocytopenia, markedly granulocytic leucopenia

and anaemia. The leucopenia and diminished immunological response renders the animal susceptible to infections especially of gastrointestinal origin. Haemorrhages varying in severity, location and clinical course are found, and may possibly be related more to vascular fragility than to the disappearance of the platelets.

Delayed effects occurring many years after apparent complete recovery from biological damage are one of the most characteristic features of irradiation damage in man; it is possible to observe these effects in terms of months in the short-lived laboratory rodents. Effects such as decreased fertility, premature ageing and radiation cataract have been noted, however, the two most important effects are the induction of tumours, malignant and otherwise and the induction of genetic mutations.

It is of course recognised that these divisions are to some extent artificial, since all these effects are superimposed (Crouch and Overman 1961) and the dominant manifestations of injury depending on such things as dose-rate and species.

The aim of the work described in this thesis was to investigate the possible parallels between the activity found in the bracken plant and the effects of ionizing irradiation, with regard to the last three syndromes.

Thus although the study is primarily on the effects of the bracken factor, the interrelationships will be considered, and it is hoped that these results will have relevance to the effects of irradiation and other chemicals, by helping to uncover the common denominators undoubtedly present.

The inflammatory nature of the changes occurring in the haemopoietic syndrome caused by bracken will be considered not merely as a function of the radiomimetic activity but more as a disturbance in the normal immunological mechanisms, and an attempt made to relate the significance of these changes to the normal and pathological states.

# SECTION I.

## THE HAEMOPOIETIC SYNDROME.

General Considerations.

A near lethal or a sublethal dose of irradiation results in lesions principally of the rapidly dividing tissues, the animal does not usually succumb to the more immediate effects of higher doses, since the direct cell damage is insufficient to produce the convulsive syndrome; nor does the radiation induced diarrhoea, with subsequent salt loss, normally occur to any great extent. The manifestations of injury, haemorrhage, anaemia, infection and malnutrition do not appear until a period of a few weeks after the dose of irradiation, however, the primary lesion is one of bone marrow and lymphoid damage and intestinal disfunction.

The effect of the bone marrow damage can be seen in the blood changes, leucopenia and thrombocytopenia develop, the severity and duration depending upon the species and the dose given. Close analysis of the changes has shown that although immediate direct damage to the circulating cells (chiefly to lymphocytes) occurs, the numbers of the platelets and granulocyte cells do not begin to fall until one to two weeks after irradiation, as a result of failure of replacement. Anaemia does occur but because of the longer circulatory life (human 120 days) of the erythrocyte, this takes longer to develop and it is probable that anaemia is not an important contributory factor since animals die or begin to recover before the level is dangerously low (Bacq and Alexander 1961).

The mechanisms of aplasia are not clear and it is not known if all cells are damaged or only the rapidly

dividing cells such as those of the bone marrow and intestinal mucosa. Probably all cells are affected but the radiation effects become obvious only in situations where rapid proliferation is necessary for the maintenance of the status quo, such as the bone marrow and the intestinal mucosa.

Patt and Quastler (1963) discuss the following three phenomena collectively responsible for the impairment of cell production.

Necrobiosis.

This mode of cell death is characterized by the rapid appearance of injury from 2 to 10 hours after irradiation and appears to be restricted to cells irradiated while in D.N.A. synthesis and attempting to go into mitosis. The morphological manifestations consist of chromatin clumping, pyknosis, a disappearance of the nuclear membrane and eventual complete loss of the chromatin structure. The effect can be reproduced by various cytotoxic drugs and appears to be responsible for the circulatory lymphocyte fall occurring immediately after irradiation; with lymphocytes the effect can be elicited by adrenal cortical hormones.

Post irradiation Freeze.

Irradiation tends to suppress most biological processes, but activities already in progress seem to be less susceptible than the preparation for them, which means that a cell about to change activities is at its most susceptible phase. If these preparations for a

change of activity pattern are blocked then the result will become manifest at the time when the change should occur. The best known example of post irradiation freeze is the inhibition of mitosis between the completion of D.N.A. synthesis and late prophase. It is of interest to note that the mitotic control of chalones described by Bullough and Rytoma (1965) appears to be mediated at the same stages of the mitotic cycle as the inhibition by irradiation according to Patt and Quastler. Bullough and Rytoma suggest that the normal effective mitotic inhibitor regulating mitotic homeostasis may be an unstable chalone-adrenalin complex, although other glucocorticoid hormones in addition to adrenalin may act to strengthen chalone function. Adrenal activity, although possibly a secondary effect associated with stress, has been observed to increase transiently immediately after irradiation. A secondary, later increase has been shown which has been implicated in the inhibition of bone marrow regeneration, although this view has not gained general acceptance.(Bacq and Alexander 1961).

However, the great variety in duration and radiosensitivity of the freezing phase indicates that there is more than one underlying mechanism, possibly all concerned with the setting up of new synthetic patterns.

Asymmetric Mitoses.

Asymmetric mitoses are possibly the result of direct chromosomal damage; the uneven separation while not often interfering with mitosis as such, leads to genetically unbalanced cells. These may be viable, may die soon after

mitosis, or even divide again before dying or maturing.

The mechanisms involved in aplasia are not clear; by the use of a radioactive label, radiomimetic drugs such as the alkylating agents have been shown to become associated only in minute amounts with the D.N.A., compared with the large amounts incorporated into protein (Schoental 1964, Bacq and Alexander 1961). Possibly for the second type of inhibition cytoplasmic mechanisms rather than nuclear injury may be the causative agent.

The eventual cause of death may be any number of different factors only indirectly related to the primary injury, indeed there may be several primary injuries all contributing to the development of the syndrome. Thus although leucopenia itself is not dangerous it is an excellent indicator of the severity of damage and the likelihood of recovery, since the development of infections etc. depends partly upon the leucocyte mediated resistance of the animal.

The following features have been considered important as causes of death.

Haemorrhages.

Haemorrhages varying in severity, location, clinical course and response may be found, the cause is probably related more to capillary fragility and damage than to the disappearance of the platelets, although this must be a contributory factor. The coagulation accelerators carried by the platelets apparently become deficient although the blood accelerators and prothrombin

remain at normal or near normal levels. The coagulation time varies, sometimes being very prolonged while on other occasions being hardly altered, this may be related to the occasional release of circulatory heparin. Massive platelet transfusion has been shown to decrease the internal haemorrhages in dogs.

Bone marrow haemorrhage has been put forward as an important contributor to overall damage (Fliedner et al. 1961); it develops before apparent thrombocytopenia possibly as a result of injury to the marrow sinusoids. Schofield et al. 1963 report that bone marrow haemorrhage was related to fatality in irradiated monkeys (800r), in general in the absence of other haemorrhages the monkeys with the most haemorrhagic marrows died the earliest while haemorrhage was absent in the monkeys that had reestablished haemopoiesis. Infection and ulceration especially of the gastro-intestinal tract may aggravate and accelerate the petechial haemorrhages, but in general although haemorrhages are seen at autopsy in many sites, they are usually insufficient to be the primary cause of death, however, occasionally a large or critically sited haemorrhage may be responsible.

Infection.

The susceptibility to infections appears to be the most important problem associated with the syndrome; the leucopenia and injury to the lymphoid tissues inhibits both the phagocytic capacity of the animal and its ability to form specific antibodies. The gastro-intestinal damage renders the animal susceptible to generalized bacteraemia originating from this site. Automatic antibiotic therapy

in the opinion of many workers is absolutely essential while others advocate only symptomatic treatment. The modification of injury by autologous bone marrow in monkeys (800r) has been shown to be ineffective if automatic continuous antibiotic therapy was not given (Schofield et al. 1963) and it is possible that antibiotic alone (Crouch et al. 1961) can promote recovery or protect the animal until bone marrow regeneration can occur.

Malnutrition.

The artificiality of division into gastro-intestinal syndrome and haemopoietic syndrome adopted in this thesis becomes evident when the gastro-intestinal damage is consider since the injury to this organ can result in an immediate salt loss or failure of function occurring later; depending on the species, dose and subject to individual variation.

The 1000r irradiated dog shows the features of the immediate gastro-intestinal damage after 3-5 days, in contras to the 450r irradiated dog which shows loss of appetite, vomiting, diarrhoea and loss of weight after about two weeks when the gastro-intestinal ulcers form, at this stage the gastro-intestinal tract is often paralysed and distended with a large content of matter. As well as bacterial attack at this site the lowered digestive capacity may contribute very significantly to the syndrome.

The absorption and metabolism of food after irradiation appears to be complex and shows species variation (Bacq and Alexander 1961), forced feeding even of predigested food may be of little use because of the loss of function of the intestinal mucous membrane and in rats increases the death rate. Possibly there are two separate

effects since sublethally irradiated rats (400r) often show loss of weight in two stages, the first immediately after irradiation and the second some twelve days later continuing about 12 days. The first effect may be due to bile flow dependent salt loss (See Section II.) and the second mediated at least partly by absorptive failure and bacterial attack.

Parenteral feeding should probably be adopted in the form of electrolytes, protein hydrolysates etc. or simply in the form of blood or plasma.

In some species ( e.g.mice) death often occurs after the apparent regeneration of the intestinal epithelium in others, man for example, anatomical damage in the form of intussusceptions or infarctions may be important if regeneration is slow.

In summary, it may be said that between species or even individuals the relative importance of the particular manifestations of injury may be different, in man for example the electrolyte, water and food absorption may be altered a great deal or sometimes very little (Bacq and Alexander 1961).

Bone marrow regeneration too appears not to be consistent but can apparently take place from very depressed levels, provided other factors are favourable. Recovery may depend not so much on bone marrow regeneration but absence of infections, haemorrhage and other complications and Crouch and Overman (1961) found that even with a functioning haemopoietic organ and the recovery of the peripheral blood picture, the animals died apparently as a result of intestinal lesions.

Bone marrow and spleen transplantation has been successfully used but suffers from the possibility of a secondary syndrome if autologous or isologous cells are not used. The secondary disease may be either a result of graft rejection by the regenerating host or a similar immunological reaction of the graft cells against the host tissues. The symptoms of this latter type of secondary disease seem to suggest that host intestinal mucosa may be destroyed by antibodies from its graft. It would seem that unless immunologically acceptable cells were available the desirability of grafts would depend on whether the host had any regenerative powers. Homologous bone marrow may be actually harmful if the dose of radiation has not been high enough to destroy the immune response, since the additional immunological stress may kill irradiated animals that would otherwise have lived (Bacq and Alexander 1961).

Bacq and Alexander point out that all the work on haemopoietic cell transplantation has not yet excluded the possibility of a chemical factor which may favourably influence symptoms of irradiation other than the damage to blood forming organs (Jacobson 1952). Post irradiation treatments with nucleic acids may be beneficial but much of the work done on this aspect appears to be contradictory.

Alkylating agents and other radio-mimetic chemicals.

The similarity of the effects of irradiation and the alkylating agents and other radio-mimetic chemicals suggests that similar early mechanisms are involved in both, although it would be erroneous to suggest a similarity of mode of action based on the similarity of the end results Nevertheless at some point in a complex chain reaction, the two possibly different pathways converge.

As with irradiation damage the possibility of a primary lesion in the nuclear D.N.A. is open to some doubt since *in vivo* experiments have not revealed the combination of alkylating agents with D.N.A. and the bound material has usually been found linked to proteins. (Schoental 1964, Bacq and Alexander 1961). While many of these agents produce D.N.A. crosslinking *in vitro* they may do so only in concentrations very much greater than that required to produce radio-mimetic effects *in vivo* . This would agree with work on irradiation where *in vivo* studies or studies on whole cell nuclei has, with few exceptions, not revealed immediate damage even with very heavy doses (25,000r); however, even 1000r produces delayed changes possibly due to secondary effects (Bacq and Alexander 1961).

Alkylating agents are capable of reacting at pH 7 with the carboxyl and terminal amino groups of proteins as well as the phosphate and amino groups of nucleic acids. The possibility of these agents and irradiation having an effect on intracellular barriers leading to cell damage should be considered, since these structures contain phosphat as well as other groups capable of being alkylated. This could lead to important changes with both internal and

other substances. Possibly leakage of enzymes across the nuclear membrane is responsible for the nuclear changes, and in a similar way membrane alterations could lead to the active amine release and the interference of nerve function seen after irradiation and nitrogen mustards (Graef et al. 1948), as well as causing the leakage of normally intracellular large molecular weight substances which could then become probably toxic or antigenic.

Mitochondrial enzyme release has been shown to take place after irradiation (Bacq and Alexander ), and possibly the D.N.A.ase content of the mitochondria is the cause of the nuclear damage. It is of great interest to note that Russian workers (Klemparskaya et al. 1961) were able to show changes similar to post irradiation effects, by injection of homologous intestinal mucosa mitochondria; and they emphasize the importance of auto-sensitization, possibly to this fraction, in the pathology of irradiation.

The alkylating and other radio-mimetic agents show variation in the type of lesion they induce, in rats low doses of the nitrogen mustard, ethyline imine and epoxide type of compound imitate the lymphoid or immediate necrobiotic effects of irradiation, while the Myleran type imitate the more delayed (10 day) myeloid effects. At higher dose levels these differences largely disappear. These agents are also radio-mimetic in that they appear to induce the immediate convulsive syndrome and the 3-5 day gastro-intestinal syndrome.

Effects of irradiation on the Bovine.

The calf, by comparison with other species appears to be highly sensitive to irradiation, the L.D. 50 lying somewhere between 150r and 250r (Schultze et al. 1959). Calves receiving this dose of gamma radiations developed acute hypoplasia of the bone marrow and a haemorrhagic syndrome; deaths occurred fourteen to twenty days after irradiation.

At dose rates above 350r, gastro-intestinal deaths become prevalent. Calves exposed to 600r whole body gamma irradiation showed a mean survival time of 9.9 days (Rosenfeld 1958) with pronounced gastro-intestinal damage, and showing the diarrhoea and anorexia characteristic of the gastro-intestinal syndrome.

These workers and Brown (1962) describe post mortem findings similar to those described for other animals, with the cellular destruction particularly of the lymphoid and haemopoietic organs, and damage to the gastro-intestinal mucosa; all characteristic of the haemopoietic syndrome. Widespread petechial haemorrhages particularly of the viscera and lungs were frequently observed, blood tinged faeces were prevalent one week following irradiation especially in the animals receiving the higher doses. Pyrexia was observed in the animals immediately after irradiation, and a second rise in temperature occurring 2-3 weeks later was indicative of a fatal outcome, usually about 5 days after the onset of fever. Bacteraemia was not thought to contribute significantly to the syndrome.

The blood changes observed were typical of irradiated animals. An immediate drop in lymphocytes occurred and after an initial neutrophilia, was followed by a more delayed granulocyte fall, the granulocytes being at their lowest 15-20 days after irradiation.

The platelets started to fall after 7 days and were at their lowest after about 21 days and Brown (1962) found that no animal whose platelets fell below 40,000 / cu.mm. survived.

Two calves exposed to 150r (Schultze et al. (1958) developed a severe blood dyscrasia between the twentieth and the thirtieth day from which they recovered. This is interesting in that it seems to be more comparable with the effects of bracken ingestion at the levels normally consumed.

Effects of D.C.V.C.

Stockman (1916) observed that cattle fed on Soybean oil meal, pre-extracted with trichloroethylene to reduce its high oil content, developed a severe and frequently fatal haemorrhagic disease.

The agent responsible for the condition was shown to be S-(trans-dichlorovinyl)-L-cysteine(D.C.V.C), a product of the interaction of the trichloroethylene and the L-cysteine in the meal (McKinney et al. 1957). A similar compound prepared from trichloroethylene and reduced glutathione was shown to have somewhat less toxicity with respect to the bovine.

The haemorrhagic syndrome produced in the bovine

is very similar to bracken poisoning, in that the changes take longer to develop than after irradiation. At the dose levels of D,C.V.C. used by the authors, bloody faeces and petechial haemorrhages with subsequent pyrexia were observed after the twentieth day following the commencement of continuous dosage. The blood changes again are very similar, with very rapid falls in platelets and granulocytes at about 20 days, resulting in complete absence of these cells by 25-30 days; this is accompanied by a more gradual and less complete fall in lymphocytes.

Later experiments have shown that the syndrome can be produced by the intravenous administration of 3 mgs, of D.C.V.C. / Kg. body weight in one day, in 3 divided doses, the effects become apparent after 3 weeks. (Schultze personal communication).

Comparative studies on the effects of the trichloroethylene extracted feeds have shown that the calf is much more susceptible to the aplastic anaemia than all the other domestic and laboratory animals tested. (Pritchard et al. 1956. Hill et al. 1956. Hanson et al. 1956).

Bovine Bracken Poisoning.

The bracken fern Pteridium Aquilinum has a world-wide distribution as an occupier of marginal land. It is fairly easily controlled by cutting, and because of this usually covers land where because of difficult topography this has not been possible. Chemical methods of control have not as yet proved effective, since the plant is capable of regeneration from the underground rhizomes, because of this both cutting and spraying need to be continued for at least three years before eradication can be achieved.

The marginal hill land on which the plant is prevalent is used in this country mainly for the production of store cattle and for sheep; it is among the young cattle that chief losses due to bracken poisoning occur, losses in the sheep are not so common and appear to show regional or breed variation. Very heavy losses have been observed in Swaledale-Scottish Blackface in the North Riding of Yorkshire (Parker and McCrea 1965), and also in Australian Merinos, but no losses have been reported in the Welsh mountain breed.

The bovine syndrome has been produced many times in the laboratory and the pathology extensively studied by Evans W.C. et al. 1951, 1954 and 1958, and Naftalin and Cushnie 1951, 1954 and 1956.

The essential lesion of the bovine syndrome is bone marrow damage, detectable two weeks after the commencement of a bracken-containing diet. The most primitive cells of the myeloid and erythroid series and the megakaryocytes are principally affected; terminally the

marrow may be almost devoid of haemopoietic cells and in many sections only a haemorrhagic stroma is visible. This often contains plasma cells (Evans I.A. personal communication).

The changes in blood cells can be seen from the animals described in this thesis. The falls take place remarkably consistently, twenty days following commencement of dosage, whether the animal subsequently recovers or dies; although it has not been possible to do many comparative experiments with different dosage levels, because of the large variation in bracken activity and the possible variation between calves. The platelets seem to be the most sensitive indicator of bone marrow damage, while the lymphocytes seem to be resistant and usually fall only gradually. Terminally when the bone marrow is completely aplastic the circulatory white cells consist entirely of lymphocytes with virtual total absence of platelets. The erythrocytes do not fall so significantly, until massive haemorrhages occur, probably because of the much longer cell life (about 120 days).

The most prominent feature of the pathology is the widespread petechial haemorrhages seen all over the body but especially in the mucous membranes; the intestine is usually badly affected and blood and mucous generally become visible in the faeces after about 25 days feeding. Very high temperatures are observed terminally, the pyrexia being higher (107°F) than that usually observed with bacteriological infections (105°F). Bacteraemia of gastrointestinal origin is probably important in untreated field cases, but has not been shown to be a feature of laboratory experiments where widespan antibiotics were given.

Attempts to induce the bovine syndrome in species other than sheep have not been successful. Sheep seem to be relatively resistant (Moon and Raafat 1951, Moon and McKeand 1953) and may show considerable breed and individual variation. Non-ruminants are affected by the powerful thiaminase activity also found in bracken (Evans W.C. et al. 1958) but cattle and sheep, because of their rumenal synthesis of $B_1$ are resistant to the enzyme.

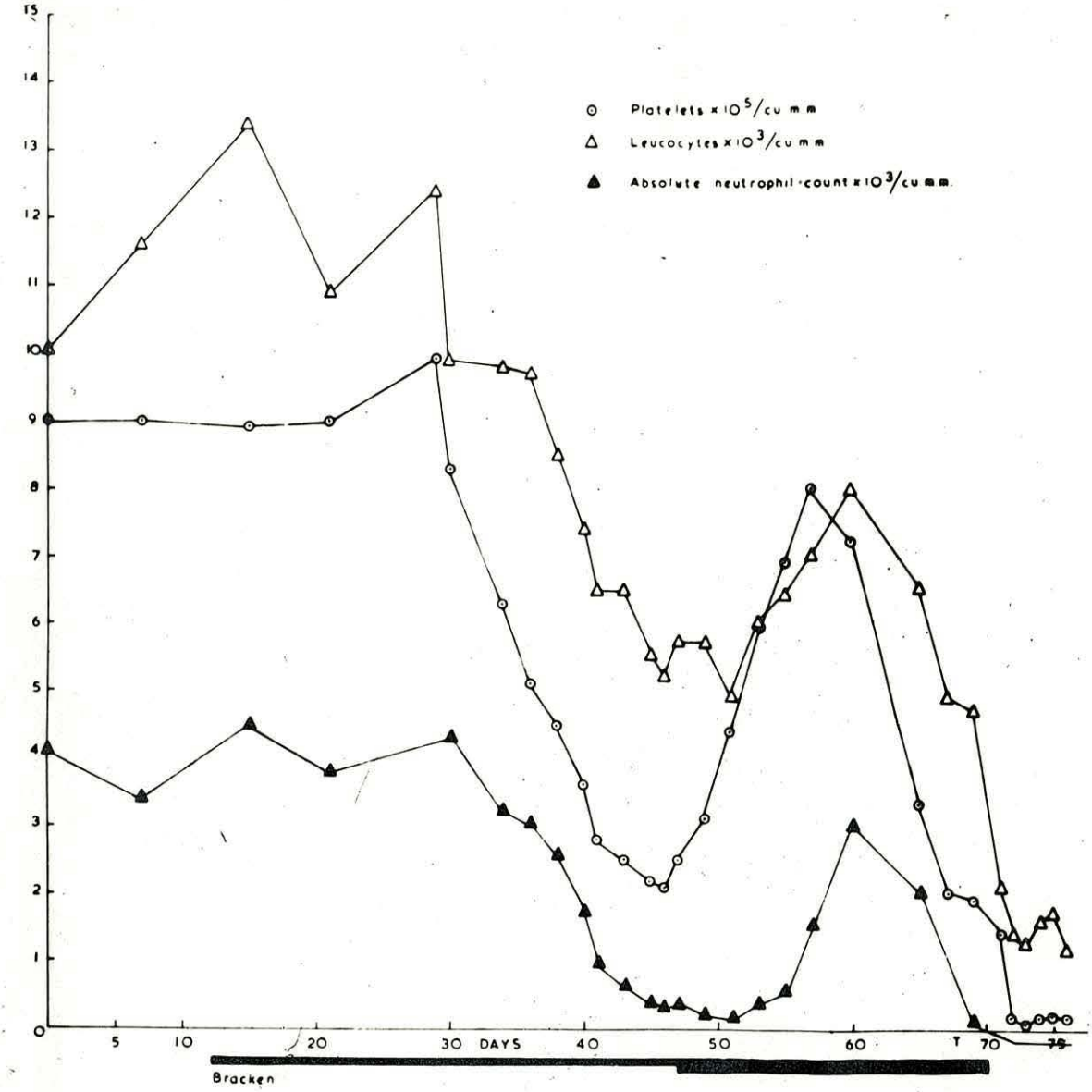
It is not possible to give meaningful comparative figures on the amount of bracken required to kill a bovine since the material shows great variation, for example Evans W.C. et al. 1961 found that ½ lb. of dried rhizome / day for 25 days was sufficient to kill a calf, while a similar calf described in this thesis (Red Friesian) showed only transient changes when given 1000 grams (2¼ lbs) / day of a dried rhizome collected at the same time of year. However, with an active sample a dose rate of 1000 grams / day of dried June frond is usually sufficient.

Work indicating Mast Cell participation.

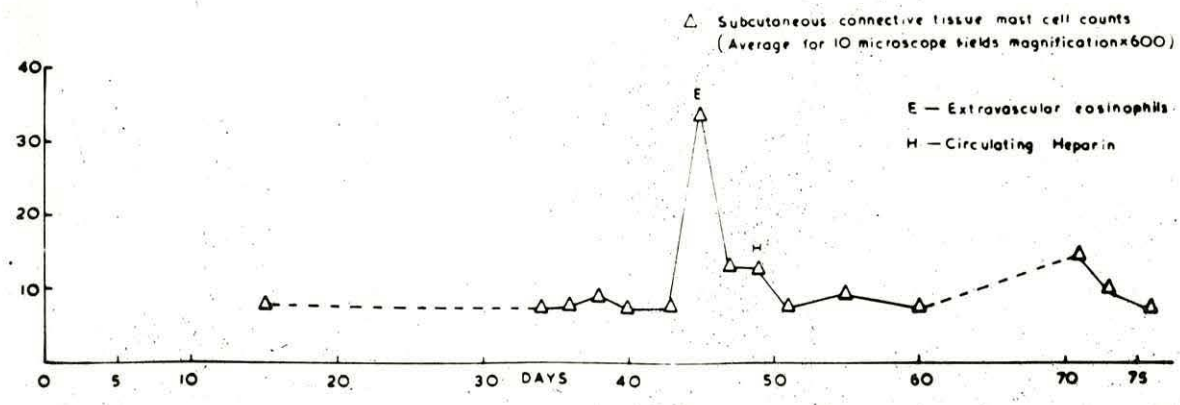
The involvement of mast cells in the syndrome of bovine bracken poisoning was first considered by Evans and Howell (1962). It had been noticed (Evans I.A. 1957 unpublished) that during the terminal stages of bracken poisoning, smears prepared for differential counts did not spread evenly in the manner of a normal sample but presented a blotched and streaky appearance, as if the slide were greasy.

Although the level of fibrinogen in the plasma is increased (Heath and Wood 1958), Howell (1962) showed that the streaking might be due to a small altered fraction, which ultracentrifuge readings showed to have a higher molecular weight than the main body of fibrinogen. The appearance of this aggregated component and the onset of streaking coincided with the appearance of circulatory heparinoid anticoagulant. Evans and Howell 1962 tried parenteral Toluidine Blue therapy to attempt to reverse the anticoagulant effects of the heparin. Other workers have shown that large molecular weight heparinoid acidic polysaccharides have a strong affinity for fibrinogen, forming similar reversible polymers (Godal 1961, Edsall et al. 1955, Morrison 1946 and Thomas et al. 1954).

The release of heparinoid anticoagulant, in abnormal amounts into the circulation, was possibly due to depolymerization of the ground substance (Blumberg and Ogston 1957), to endotoxin induced Schwartzman reaction - like effects on the leucocytes( Thomas et al. 1955 a & b), or was of platelet origin (Kerby and Langley 1959).




BLOOD COUNTS




TISSUE MAST CELL COUNTS.

FRIESIAN CALF

Fig. 1.

However, the most likely source seemed to be tissue mast cells, and it was decided to attempt to assess the involvement or destruction of these cells during the course of acute bovine bracken poisoning. The results obtained, using the technique developed by Dr.I.A.Evans, are represented in Fig.( 1 ) from Howell(1962).

In view of these findings, the aim of the work described in this thesis was to confirm these results and to investigate more fully the role of the mast cell in bracken poisoning, to study the function of the mast cell in this and other states and to try to see if these findings were relevant to irradiation effects.

The Mast Cell. (A general survey).

The first description of tissue mast cells was made by Paul Ehrlich (1877) using the new aniline dyes then being produced by the German chemical industry, he noted that the cells although widely distributed were more numerous in well vascularized connective tissue. Jorpes (1937) showed that the characteristic metachromasia of the granules, after staining with toluidine blue, was due to the highly sulphated polysaccharide heparin, while Riley and West (1959) were the first to show that the mast cells were the chief source of tissue histamine; in addition, Lagunoff and Benditt (1963) indicated the presence of powerful proteolytic enzymes in the mast cells.

It is true to say that while the pharmacology of the inflammation-mediating substances of mast cell origin has received considerable attention, the functional role of the mast cell in relation to the connective tissue has only just begun to be studied.

Riley (1962) postulated the muco-polysaccharide cycle in which highly sulphated mucopolysaccharide was built up by the mast cell and shed as granules which were phagocytized by the fibroblasts; the histamine content of the mast cells acting as a stimulant for phagocytosis and digestion. The mesenchymal cells were thereby stimulated to form their own specific mucopolysaccharide, and to contribute in this way to the formation of new extracellular ground substance. Once this has served its purpose, it may in turn be broken down, rebuilt and stored in a highly sulphated form in the mast cell granules. West(1962) indicated that 5-hydroxytryptamine, another important mediator of inflammation, which is contained in mast cells of some species, notably the rat and mouse, exerts an even more powerful phagocytic effect than histamine. In this context, Miller and Whitting (1965) showed that mast cell proliferation was concerned with the formation of new tissue during wound healing.

Whether this cycle is a normal process is not clear, none of the samples taken during the control periods of animals described in this thesis, have revealed phagocytosis of mast cell granules or even the presence of authentic shed mast cell granules, however, it is possible that the material is shed in a soluble or otherwise altered form.

Mast cells are known to be the mediators of the anaphylactic reaction and other manifestations of immediate hypersensitivity (Mota 1963, Keller 1962), the mast cell damage, and subsequent release of contents, apparently being caused by antigen-antibody reaction on the cell surface. This is probably not so much a physical effect as an energy-requiring trigger mechanism (Mongar and Schild 1962);

A phospholipase enzyme possibly concerned, may be the producer of Slow-reacting-Substance (S.R.S.), another mediator of anaphylaxis and possibly the inflammatory sequence, (Uvnas et al. 1962, Uvnas 1963).

The important concept of Boyden (1964), suggesting that inflammation following cell injury is at least in part the consequence of an auto-immune reaction, could indicate a fundamental role for the mast cell. He visualizes the normal presence of auto-antibodies to cell components, and inflammation after cell damage being a result of the interaction of the damaged cell components, and the antibodies already present.

Pure suspensions of mast cells have been obtained in tissue culture from the differentiation of lymphoid cells from the mouse thymus (Ginsburg and Sachs 1963); and although Walker (1964) showed that neonatal thymectomy did not reduce the size of the mast cell population, it is probable that the small lymphocyte, the plasma cell and the mast cell have the same common origin. If this is so then all must be assumed to possess the same genetic potentialities of immune reactivity (Burnet 1964).

If this concept is borne in mind, then the function and many of the effects of mast cell involvement can perhaps be understood. Auto-immune processes (discussed later) are known to occur after many injuries such as surface burns (Kantor et al. 1965), although antibodies to normal skin are known (Boyden 1963), and it is possible that the inflammatory changes of repair are dependent upon the presence or initiation of these processes and consequent mast cell involvement. If plasma cells can be stimulated, why not mast cells? Numbers are known to increase in Nippostrongylus brasiliensis infected rats

(Wells 1962), and it appears that the mast cells play an important part in the cellular reaction to parasite infection Localised increases in mast cell numbers during chronic inflammation have been reported by Hlavecek and Lodja 1963, although during acute inflammation numbers decrease, probably as a result of disruption. Both these authors and Wells (1962) draw attention to the importance of eosinophil response, and interpret it as playing a role in counteracting the harmful effects of released histamine and in the development of subsequent resistance.

Csaba et al.(1961) state that the mast cell reaction and the plasma cell reaction to tumours are but different cytological manifestations of the organism's defence; this agrees with the concept of Higginbotham (1963) who regards the mast cell as the non-specific mediator of resistance, compared with the plasma cell as the specific mediator. He suggests that heparin is a preformed type of protective substance with a localized action and general non specific activity, whereas the action of induced antibody is systemic and restricted to homologous antigen.

Androgenic and Oestrogenic hormones have been shown to decrease the numbers of mast cells in the myometrium of the human Cervix Uteri (Iverson 1960). The effects of adrenal cortical hormones have been observed, A.C.T.H. in man and cortisone in rats, causing a decrease in the numbers of normal tissue mast cells (Asboe-Hansen 1950, Cavallero and Braccini 1951); in man there is a rapid rise in the circulating heparin-like substances after administration of cortisone (Smith et al. 1950). Bloom (1952) observed the regression of canine mastocytomas after the administration of cortisone.

These results are highly significant in view of the inhibiting effect of corticosteroids on antibody production, inflammation, hypersensitivity etc. (Humphrey and White 1964). Mast cells are known to increase during carcinogenesis (Stolk 1963, Simpson 1963, Riley 1959) and in the vicinity of induced, homologous and heterologous tumours (Csaba et al.1961); this is interesting in view of the immunological aspects of cancer discussed in section III of this thesis.

Smith and Lewis (1953) studied the effects of irradiation upon the mast cells of the hamster and mouse, they concluded that the decrease and degranulation occurring markedly 7-10 days after irradiation, could at least in part account for the post-irradiation clotting defect, however, the results of earlier workers conflict and in view of the results described in this thesis the anomalies are possibly due to inadequate sampling and the rapidity of fluctuations.

The literature concerning mast cells is vast and no attempt has been made to present a comprehensive or even a balanced survey, whether inflammation is good or bad will not be argued here, but whether the role of the mast cell is beneficial or adverse would seem to depend upon the degree of reaction and the capabilities of the tissues to respond. Perhaps, like so many other phenomena, it is possible to have too much, or too little of a good thing.

Histamine.

The classical demonstration of histamine by the Schultze-Dale reaction of isolated guinea pig gut is too well known to warrant description. The main source of the

histamine responsible in vivo for the manifestations of hypersensitivity is the mast cell; in some species, notably the rabbit, the platelet is another important source.

Local release of histamine appears to initiate the lowering of vascular flow and the increased permeability seen in inflammation (Spector and Willoughby 1963); but West (1962) has put forward the idea that histamine also has the function of activating the enzymic formation of bradykinin. This nonapeptide has been shown to produce vasodilation, to increase capillary permeability, to cause leucocyte accumulation and to produce pain (Edery and Lewis 1962). Possibly the plasma kinins are responsible for the more delayed vascular changes and for their prolonged maintainance.

Histamine, 5-hydroxytryptamine and bradykinin, the three agents which can together produce the phenomena constituting the typical inflammatory picture, are all antagonised by eosinophils although only histamine is chemotoxic for eosinophils (Archer and Broome 1963). Wells (1962) found that a pronounced eosinophil response constituted an important part of resistance to parasite infection. Holman (1953) describes an "eosinophilia" of recovery" during the changes in the leucocyte picture following tissue injury. Spiers (1964) assigns to the eosinophil the role of carrier of antigenic material to the series of cells able to digest the antigen and to produce specific antibody. (Macrophages, plasma cells and lymphocytes).

Cortisone and hydrocortisone have been shown to retard the regeneration of mast cell histamine and 5-hydroxytryptamine after depletion with the drug 48/80

and also to produce a decrease in both mast cell and non-mast cell histamine in normal rats (Carr and Marshall 1962), cortisone has also been shown to reduce eosinophil numbers (Code and Mitchell 1957).

Spencer and West (1963) have summarized the sensitivities of rats and mice, in different hormonal states, to histamine; in general there is antagonism between the adrenal cortex and the thyroid, but the adrenal cortex appears to be the more important regarding a direct effect on histamine metabolism. High levels of glucocorticoids decrease the animal's histamine sensitivity.

Only the free histamine released from the mast cells is pharmacologically active and this is quickly broken down in most species by the enzyme diamine oxidase (Schayer 1959); some is excreted, and Adam (1950) stated that the urine and not the blood should be assayed when a pathalogical histamine release is suspected. However, the assay of urine by the quick fluorimetric method, used in this thesis, is difficult since there is interference by the high concentration of ammonium ions.

Cysteamine, one of the best protective agents against radiation injury, is also an inhibitor of diamine oxidase activity, and Marco et al. (1962) suggest that a temporary increase in histamine concentration, as a result of the drug, may result in a beneficial effect against radiation injury. Histamine itself is known to be a good radioprotector (Bacq and Herve 1952). However, cysteamine may act by protecting the -SH groups of the histamine releasing enzymes (Spector et al. 1963).

Once again, the beneficial or harmful effects of inflammation are not clear since Field et al. (1964) were

able to reverse the effects of a near-lethal dose of nitrogen mustard with a single intra-peritoneal injection of anti-histamine drugs, either just before or just after the nitrogen mustard. The resultant leucopenia was also less in extent and duration. Desensitizing drugs such as Novocaine were used with great success by Klemparskaya et al.(1961) in counteracting the effects of lethal doses of irradiation (1000r) in rabbits.

It was felt that the blood histamine levels of bracken poisoned calves ought to be followed in parallel with the tissue mast cells, in order to gain a better understanding of the condition, and design possible therapy. The gut damage seen in bracken poisoned calves is possibly partly due to histamine, since histamine has been implicated in the production of gastric ulceration (Hunt and Hunt 1957)

Heparin.

Mast cells are the source of heparin or more correctly the heparinoid type of highly sulphated polysaccharides. The spectacular release of heparin from mast cells in the canine liver and entry into the peripheral blood causing incoagulability, is an unusual hypersensitivity or shock effect. Almost certainly in this case it is due to the peculiarities of the dog's circulatory system (Riley 1959); and it is now generally accepted that heparin is not the "natural anticoagulant" but that its main function lies in the tissues. The presence of circulatory heparin in bracken poisoning should probably be thought of as rather an unusual overspill phenomenon. Riley (1962) has postulated the mucopolysaccharide cycle, however, it is possible that this cycle becomes important only in tissues where a high

degree of repair or building is taking place. Heparin, unlike histamine, is not released without mast cell disruption and the heparin does not produce its effects unless the granules themselves are disrupted (Archer G.T. 1961). In the normal bovine, detectable mast cell disruption not attributable to technical artefact does not appear to take place, although it is unwise to be categorical on this point.

The mast cells synthesise heparin, and the maturity of the cell can be deduced from the degree of sulphation of the granules and consequent metachromasia (Radden 1962). Although Loomis (1961) has stated that mast cells were able to take up $S^{35}$ labelled heparin as such, it is probable that the localisation of radioactivity was attributable to the uptake of $S^{35}$ labelled sulphate resulting from the rapid catabolism of injected heparin (Day et al. 1962). Spotter et al.(1963) were able to show the NAD enhanced incorporation of $S^{35}$ sulphate into both low molecular weight substances, and into heparin in mast cell derived cell-free systems, The rate of sulphate incorporation into peritoneal mast cells is greater in cells of young rats than of old rats (Guidotti and Spinelli-Ressi 1964) possibly indicating that the mucopolysaccharide turnover is greater where tissue building is rapid.

The replacement of mucopolysaccharides is inhibited by cortisone, hydrocortisone and irradiation (Foster 1963 and Gerber et al. 1962), and it is possible that these effects are mediated by mast cell inhibition. Cortisone and hydrocortisone have been shown to inhibit connective tissue growth in the healing of wounds. The mucopolysaccharide cycle may therefore be at least partially dependent

upon mast cell activity and numbers, and in times of rapid growth or repair after damage, a mast cell response may be of crucial importance.

Clotting defects produced in rabbits by irradiation and nitrogen mustards were observed by Jacobson et al. (1948); the defects although related to the decrease in platelets, were attributed to the presence of circulatory heparinoid substances and were reversible with anti-heparin therapy. Heparin, although having a very strong anti-thrombin effect, also has an anti-thromboplastin activity (Ollendorff 1961). The effects are antagonized by platelets, especially if the platelets are disintegrating (Godal 1962); so it is possible that bracken poisoned calves with low platelet levels are more susceptible to heparin. Heparin does not inhibit the fibrinolytic activity of bovine plasmin but does inhibit the activation of plasminogen by strepto-kinase (Holmans 1962).

Heparin has an effect as an inducer of a lipoprotein lipase activity at concentrations less than needed as an anticoagulant. Monkhouse and Baker (1963) found that although the level of skin heparin was decreased by irrad-iation, the post-heparin clearing factor lipase activity was increased; the significance of this is not clear, although irradiation induced defects of fat metabolism have been reported.(Goldwater and Entenman 1956 and 1957).

The possible harmful effects of heparin must be balanced against the beneficial, and Higginbotham (1963 and 1959) has suggested that the mast cell has an important local action in clearing toxic or potentially toxic foreign material. The acidic polysaccharide can be shown to form complexes with noxious substances and to stimulate their

ingestion by the fibroblasts of the loose connective tissue. It is possible that this process or micellophagosis is a natural process which serves to maintain a functional or sub-inflammatory state of the connective tissues. The discharge of heparin may be a kind of non-specific clearing mechanism, a primitive antibody. In this connection, it is of interest to note that mast cells appear to be present throughout the animal kingdom, from sponges to man. (Michels 1938).

As well as in local tissue inflammation, mast cells have been shown to have a systemic effect in the fixation of blood-borne particles (Selye et al. 1963). Precipitation of toxic material frequently caused necrosis similar to the Schwartzman reaction, although heparin has been shown to inhibit both the Arthus reaction and the Schwartzman reaction, possibly by its anticoagulant activity preventing the vascular damage observed in both (Good and Thomas 1953).

The protective action of heparin has also been demonstrated in experimental immune nephritis, induced in rabbits by the injection of heterologous nephrotoxic serum (Halpern et al. 1965). They suggested that the action was possibly related to inhibition of the local effect of complement during the antigen-antibody reaction.

Proteolytic enzymes.

The presence of multiple proteolytic, or more correctly esterase, enzymes in mast cells has been demonstrated by their action on synthetic substrates (Lagunoff and Benditt 1963, Ende et al. 1964 ) and human mast cells and dog mast cell tumours have been shown to possess a very high fibrinolytic activity

(Ende and Auditore 1961). It is possible that these enzymes play an important part in the localised breakdown of noxious substances, in a manner analogous to the suggested role of the enzymes generated from complement by Ag-Ab. reaction. These enzymes if released from mast cells at the same time as heparin and histamine, may play an important role in the tissue damage observed in bracken poisoned calves.

Fibrinolytic activity was observed by Cronkite (1950) in irradiated animals, however, this may be due not so much to an increase in enzyme levels as a fall in plasmin inhibitor, similar to the one observed by Kochlaty et al. (1952) after irradiation. These observations are probably the result of non-specific stress effects, since these authors observed a transient decrease followed by an increase in both irradiated animals and starved controls. Kamel et al. 1963 postulate a dynamic equilibrium between the coagulation mechanism depositing fibrin and the plasmin fibrinolytic system constantly removing it; a normal response to stress, according to these authors, is a transient rise in fibrinolytic activity followed by an increase in anti-fibrinolysis and an ultimate rise in fibrinogen. It is perhaps significant that a large increase in fibrinogen occurs during the bovine bracken syndrome. (Howell 1962).

The role of protease enzymes in allergy, hypersensitivity and inflammation appears to be very complex, many of the enzymes although remarkably active in hydrolyzing synthetic ester substances, show no activity on protein substrates, possibly because they are highly specific, acting in vivo on a single or a small number of functionally active protein substrates

(Ungar et al. 1961). The relationship of the fibrinolytic system to the Kinin forming esterases is not clear although Eisen (1963) suggests that even though kinin forming factors exist which are independent of this system, some of the kinin generation is due to the fibrinolytic factors.

Austen and Brocklehurst (1961) found that the anaphylactic release of histamine by guinea pig lung in vitro was prevented by chymotrypsin substrates and inhibitors but not by trypsin and some other enzyme substrates and inhibitors. Perera and Mongar (1963) have quite clearly differentiated this functional chymotrypsin-like histamine-releasing enzyme of mast cells from the chymptrypsin enzyme content of the cells. However, Shore et al. (1962) showed that the release of histamine from rabbit platelets was blocked by p-tosyl arginine methyl ester, a substrate of trypsin-like enzymes.

Thus in summary, the localized effect of proteolytic enzymes may be important in tissue injury, since synthetic inhibitors of proteolytic enzymes have been shown to be effective in suppressing systemic anaphylaxis, acute endotoxaemia and stress shock (Zweifach et al. 1961). Also a natural polypeptide, having the property of inhibiting several proteolytic enzymes including chymotrypsin, trypsin and plasmin has been shown to inhibit the Schwartzman reaction. (Halpern 1964). Whether these inhibitors prevent direct damage to the tissues or inhibit the release of histamine, kinin, 5-hydroxytryptamine and bradykinin etc. is not clear, and the fibrinolytic or destructive system cannot be differentiated entirely from the functional system.

# METHODS

## Histamine.

The method was essentially that of Shore, Burkhalter and Cohn (1959), with some of the modifications of Kremzner and Wilson (1961). It depended on the condensation of histamine with ortho-phthalaldehyde in alkaline conditions; forming a fluorescent product which was subsequently stabilized by the addition of acid. The histamine was extracted with n-Butanol from deproteinized extracts under alkaline conditions, where most of the interfering compounds do not retain their positive charges and are not extracted. Residual traces of these compounds, mainly histidine and histidyl-histidine, were removed by a subsequent washing with dilute alkali.

The histamine was measured in an aqueous extract of the n-Butanol by formation of the fluorophore and comparison of this with a standard source of fluorescence. Kremzner and Wilson 1961 suggested that the fluorescence should be measured at pH 2 rather than pH 0·85 since the intensity is greater and independent of pH.

## Reagents and Solvents.

1. Analar grade Perchloric acid S.G.1·54
2. Analar grade Sodium Chloride.
3. Analar grade n-Butanol.
4. NaCl saturated 0·1N NaOH solution.
5. Lab. reagent grade n-Heptane (99%).

6. 0•1N HCl.

7. 5N NaOH solution.

8. 1% solution of ortho-phthalaldehyde in Methanol. (O.P.T.reagent), the reagent is stable for at least two weeks if kept in a refrigerator.

9. 2•5M $H_3PO_4$ (o-phosphoric acid).

10. 5•8µg / ml. quinine sulphate in 0•1N $H_2SO_4$ diluted ten times in 0•1N $H_2SO_4$ before use.

Procedure.

Blood was collected from the jugular veins of animals into 25 ml. McCartney bottles, each containing 0•5 ml. of a solution of sodium heparin (75 mg.) in physiological saline (50 mls.). The bottles were stoppered, gently inverted ten times and cooled under the tap. The blood was then processed as quickly as possible to reduce enzymic loss.

To measure the total histamine, i.e. both cellular and free, 10 mls. of the heparinized whole blood were taken and placed in a 50 ml. polythene centrifuge tube with 9 mls. of distilled water; 1 ml. of concentrated perchloric acid was then added drop by drop while the tube contents were swirled. The tube was then allowed to stand for one hour.

After centrifugation at 4000r.p.m. for half an hour the supernatant was transferred to another polythene centrifuge tube; sodium chloride (5 grams), 5N NaOH (2mls.) and n-Butanol (30 mls.) were then added. The centrifuge tube was stoppered and shaken vigorously for five minutes. After a few minutes of centrifugation at 4000r.p.m. the

supernatant Butanol layer was pipetted off into another centrifuge tube, this was shaken for two minutes with salt saturated NaOH ( 5mls.) and recentrifuged.

The supernatant Butanol was added to n-Heptane (55 mls.) and 0·1N HCl (15 mls.) in a separating funnel, this was shaken for ten minutes and allowed to stand for ten minutes. The lower layer was then allowed to drain into a 50 ml. beaker, and boiled down to a volume of 3-4 mls. after adding a small piece of porous pot.

The residue was transferred to a graduated reductase tube and the beaker washed with 2 mls. of distilled water.

5N NaOH (0·5 ml.) followed by the O.P.T. reagent (0·5 ml.) was added by pipette and mixed by drawing and expelling. After $3\frac{1}{2} \pm \frac{1}{2}$ minute, 2·5 M. $H_3PO_4$ (1ml.) was added and mixed. The volume was then adjusted to ten mls. and the fluorescence measured immediately against the standard quinine sulphate solution (0·58 µg./ ml.).

The free histamine in the plasma was measured in the same way except that 4 mls. of distilled water instead of 9 was added as the first step, the plasma having been obtained by centrifuging at 3000r.p.m. for twenty minutes.

Fluorimeter.

This consisted of a Hilger-Spekker fluorimeter with a mercury vapour lamp as excitation source. This light was passed through a filter (H556) into the base of the $10cm^2$ x 1cm. cell containing the fluorescent solution. The fluorescence was detected on sensitive photoelectric cells placed adjacent to the sides of the

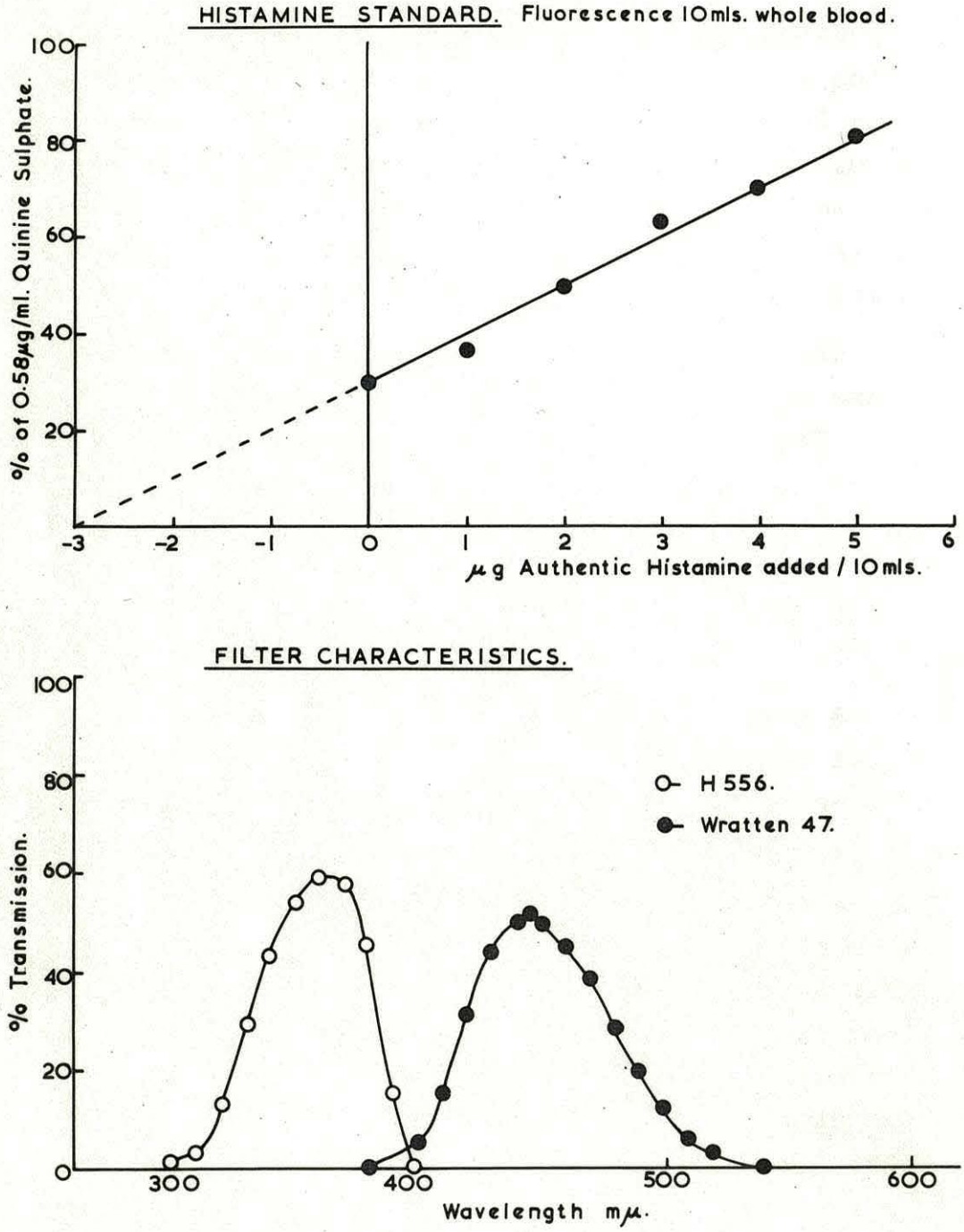

HISTAMINE STANDARD. Fluorescence 10mls. whole blood.
% of 0.58μg/ml. Quinine Sulphate.
100
80
60
40
20
-3
-2
-1
0
1
2
3
4
5
6
μg Authentic Histamine added / 10mls.
FILTER CHARACTERISTICS.
% Transmission.
100
80
60
40
20
0
H 556.
Wratten 47.
300
400
500
600
Wavelength mμ.

Fig. 2.

cell behind the Wratton No.47 filters at right angles to the incident light.

Shore, Burkhalter and Cohn reported that the excitation spectrum of the Histamine-O.P.T. product has a peak at 360mμ while the fluorescence spectrum has a peak at 460 mμ. It can be seen (Fig. 2 ) that the filters selected corresponded very closely to those values while the very small overlap (plus the positioning of the photo-cells) ensure that the incident light is not measured.

The output of the photo-cells was detected with a Wheatstone Bridge arrangement crossed by a mirror galvanometer. The sample to be determined was placed in the light path and the resistance altered until the galvanometer registered zero, the standard was then substituted and the galvanometer brought back to zero by reducing the excitation light. The amount of light required was read on a calibrated drum.

A graph of the drum reading against identical samples of blood having graded amounts of histamine already added before being put through the procedure is shown in Fig. ( 2 ).

A sample of normal blood usually gave a drum reading of 25-30% of the standard; an extrapolation of the graph indicates that if the fluorescence was due entirely to histamine the bovine blood content was about 3μg./ 10 mls. This corresponded to values of 0·25-0·36 μg./ ml. obtained by Wrenn et al. (1963) using the method of Shore, Burkhalter and Cohn. However, this is not likely to be a true value since a reversal of the order of addition of 5N NaOH and 2·5M $H_3PO_4$ which prevents the formation of the fluorescent product indicates a non-specific background of 10-12%.

This would give a true histamine content of about 1·8 µg. / 10mls., which is close to the figure of 0·165 µg./ ml. obtained by Romanelli (1949) using the biological assay with isolated guinea-pig gut.

The results obtained were plotted as drum readings rather than as absolute values.

Heparinoid Anticoagulant.

The method was based on that of Jacobson et al. 1948 and depends on the ability of the basic protein protamine sulphate, derived from salmon sperm, to neutralize the effects of the acidic polysaccharide heparin (Birkinshaw and Smith 1962). The following procedure was developed from that of Howell 1962.

Procedure for Whole Blood.

The animals were bled into McCartney bottles as described in the Histamine procedure.

Protamine sulphate (25 mg.) was dissolved in physiological saline (50 ml.) and made up freshly for each determination since the protamine sulphate deteriorates in solution. Graduated amounts of this solution were put into ten glass test tubes, 8 mm. in diameter, using a 0·1 ml. opsonic pipette, the following table indicates the amounts used.

| Tube No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Mls. P.S. soln. | ·02 | ·03 | ·035 | ·04 | ·045 | ·05 | ·06 | ·07 | ·08 | ·09 |
| µgms.P.S. | 10 | 15 | 17·5 | 20 | 22·5 | 25 | 30 | 35 | 40 | 45 |
| Heparinized blood mls. | ·25 | ·25 | ·25 | ·25 | ·25 | ·25 | ·25 | ·25 | ·25 | ·25 |

After the addition of the heparinized whole blood the rack containing the tubes was shaken gently for a minute and the tubes were then allowed to stand for twenty minutes.

At the end of this period and at subsequent five minute intervals the tubes were examined to ascertain the tube containing the most clotting activity. Since protamine sulphate as well as heparin has multiple anticoagulant activity (Ollendorff 1962), blood in tubes each side of the equivalence point has a decreased clotting ability.

A tube was considered to have clotted when the blood remained firm after the inversion of the tube, tubes on either side of the equivalence point usually exhibited a slight degree of clotting. The presence of fibrin clots could be verified by filling the tube with distilled water. The results are recorded as μ gram protamine sulphate required for the equivalence point.

The main difficulty with this method when applied to the bracken poisoned animals was that the heparinoid increases usually took place at the time the animal had few platelets. The low inherent clotting capacity made the test difficult, and valueless in the cases when the blood failed completely to clot.

Plasma Heparinoid Anticoagulant.

An attempt was made to design a test independent of the platelet "thromboplastic activity".

The following procedure was developed. Dade "Activated thromboplastin" (•025 mls.) prepared from rabbit brain (Dade Reagents, Miami, Florida.) was diluted to 0•5 mls. with Physiological saline. 0•02 mls. of this was put into ten test tubes of the same type as those used

for the whole blood test. Graduated amounts of protamine sulphate as indicated in the following table were then added.

| Tube No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Mls.P.S. | ·015 | ·02 | ·025 | ·02 | ·035 | ·04 | ·045 | ·05 | ·06 | ·07 |
| µgm. P.S. | 7·5 | 10 | 12·5 | 15 | 17·5 | 20 | 22·5 | 25 | 30 | 35 |

The heparinized whole blood was centrifuged at 3000r.p.m. for twenty minutes and 0·25 mls. of the plasma added to each of the ten test tubes. The rack containing the tubes was then shaken and allowed to stand . Tubes containing excess of protamine sulphate form a strong opaque fibrin clot within ten minutes. The equivalence point was taken as that tube, containing the least amount of protamine sulphate, which gave a clot.

There was little or no thromboplastic activity left in the plasma since tubes containing excess protamine sulphate but no added thromboplastin, could be left 48 hours without clotting. The normal reading obtained with the heparinized blood was 15-20 µ grams protamine sulphate; an increase in the amount of authentic heparin in the blood collection bottle resulted in a corresponding increase in the reading.

In both tests it was essential to ensure that all the test tubes were absolutely clean, and so after brushing with a pipe cleaner they were routinely soaked in chromic acid for twentyfour hours, before being washed in distilled water.

Proteolytic enzymes.

Proteolytic enzymes were estimated by a potentiometric method depending on the hydrolysis of synthetic substrates by the enzymes. The hydrolysis of the ester linkage of p-toluene-sulphonyl arginine methyl ester (T.A.M.e.) and N-acetyl-tyrosine ethyl ester (A.T.E.e.) by the trypsin and chymotrypsin-like enzymes respectively, produces free acids, so the rate of release of the acids can be measured by recording the amount of alkali of known strength required to maintain a constant pH (Laskowski 1955)

The reaction took place in a glass vessel with a capacity of fifteen mls. maintained at a constant temperatur by means of an outer jacket through which water from a constant temperature reservoir circulated.

The pH of the solution was measured by means of glass and calomel electrodes dipping into the solution (0·25M NaCl). The pH was maintained at a constant value by the automatic addition of 0·02N NaOH from a 0·5 ml. syringe, the solution being agitated by a mechanical stirrer. The addition of alkali was recorded.

Reagents.

1. T.A.M.e. hydrochloride (0·57 grams) solution in distille water (50mls.); 2 mls. of this diluted to 15 mls. in the reaction vessel gave a final concentration of 0·004 Molar.
2. A.T.E.e. (1·01 grams) was dissolved in Ethoxy-ethanol (37·5 mls.) and distilled water (12·5mls.); 2 mls. of this diluted to 15 mls. gave a final concentration of 0·01 Molar and a 10% solution of ethoxy-ethanol.

3. NaCl solution (0·75 Molar).

4. NaOH solution ( 0·02 Normal).

Procedure.

0·75M NaCl solution (5mls.) and distilled water (7mls.) were placed in the reaction vessel maintained at 25°C and allowed to reach equilibrium, heparinised plasma (1ml.) was then added and the machine allowed to attain a pH of 7·8, this and the very small addition of 0·02N NaOH, required to maintain this pH was recorded. After several minutes T.A.M.e. or A.T.E.e. solution (2mls.) were then adde after initial rapid addition a steady state was attained as the machine maintained pH 7·8. The amount of enzyme activity could be directly determined from the recorded NaOH addition rate.

In these conditions the rate of reaction is depende only on enzyme concentrations since the substrate present wa 60 µMoles for T.A.M.e. and 150 µMoles for A.T.E.e.; the reserve alkali was 10 µMoles.

Enzyme inhibitors.

Plasma also contains proteolytic enzyme inhibitors so an attempt was made to assess the dynamics of the enzyme-inhibitor systems.

The following procedure was adopted. 0·25 mls. of a solution of B.D.H. "Trypsin" (0·1gram) in distilled water (100 mls.) was added to the 5 mls. of 0·75M NaCl and the 7 ml of distilled water. The machine was allowed to titrate to pH 7·8 and 2 mls. of T.A.M.e. or A.T.E.e. solution added, the rate of enzyme reaction was noted from the linear portion of the graph. After allowing the machine to titrate about

eight minutes in this region, 1 ml. of plasma was added. The decrease in enzyme activity was noted.

Values for enzyme activity were taken at all times from the linear portion of the graphs occurring after the machine had restored the pH to 7·8 following the addition of fresh solutions to the mixture. The large excess of substrate ensured that the rate was dependent only on the enzyme concentration and titration to the full capacity of the alkali reserve (10 μM) did not reveal any decrease in rate

It was realized that the methods were measuring many different types of enzyme, some possibly highly specific and without action on general protein substrates. Similarly the use of crude enzyme preparations to measure inhibitor levels may be criticized, but it was felt that this method was useful in that it was suitable for quick serial estimation and that large changes would be seen which could be related to the other parameters studied. Any indications obtained could then be studied in greater detail. Plasma rather than serum was used because during the terminal stages of bracken poisoning blood obtained from the calves often fails to clot or clots very slowly. The heparin added did not inhibit the breakdown of the artificial substrates by the B.D.H. Trypsin.

## Haematological and Histological Techniques.

All the techniques described in this section were developed by Dr.I A Evans, and the observations and figures from the use of these techniques were obtained by her. The only exceptions being the total leucocyte and platelet counts of calves 152, and 154, which were obtained by the author, and calf "Dafydd", obtained by Mrs. A.Tong.

### Subcutaneous connective tissue.

The study of events in the blood alone are unsatisfactory, in that the phenomena observed are often no more than overspills from the tissues; so in order to have an idea of the allied tissue reactions the connective tissue was studied in parallel. When the biopsies were made, they were taken immediately after the withdrawal of the blood sample, and the following procedure was adopted.

The animal's coat was clipped short on the flanks and a small area of 6 x 6 sq.ins. shaven, this and the surrounding area were then washed with soap and water, dried carefully and rubbed with 70% ethanol. 2mls. of a 1% soln. of Lignocaine in the sterile cartridges used by dentists was injected subcutaneously in the shaven area and dispersed by gentle massage. A small incision (1") was made through the outer skin and muscle layers and the subcutaneous loose connective tissue exposed. Samples of this were taken by pulling out a small piece with forceps, cutting, then placing on a piece of filter paper. As the incision could be moved quite easily over the connective tissue, samples (normally six in number) could be taken over a radius of about five inches. The incision was closed with a nylon suture and the wound sealed with an

antibiotic cream (Strypen intramammary). The wounds healed within two to three days and the suture was removed at the end of this time, no infections were ever observed in the animals described in this thesis, although during the terminal phases sterile haematomas occasionally developed.

Although it was impossible in the conditions to observe strict sterility, every possible precaution was taken

The tissue samples were spread as quickly as possibl before drying out, on to glass cover slips (No.2 7/8 sq,) and fixed overnight or longer in 80% ethanol. The spreads were then stained in Wright's stain diluted with distilled water(1:4) while the stain was in contact with the tissue, and allowed to stand fifteen minutes in covered Petri dishes (4 per dish). Further processing was carried out in the same dishes, care being taken to wash each fluid well over the preparations by means of a pipette. The steps were as follows.

1. Wash quickly with distilled water.
2. Differentiate with 0·08% acetic acid for 1 to 1½ mins.
3. Dehydrate with ethanol for 2 to 2½ mins.
4. Clear in Xylene 5 to 10 mins. or longer.
5. Mount in Depex.

Some of the tissue spreads were stained with pyronin-methyl green stain, in order to demonstrate cells rich in R.N.A. and D.N.A..

The mast cell counts in the mounted specimens were performed by noting the number of cells in a single field at a magnification of 600. Since the number of mast cells varies with the vascularization of the area, counts were performed on fields centred on a branching system of blood vessels, through a depth of 0·01mm. of

tissue. The average of 40 different fields was taken, using at least two different preparations. It was found that this method gave remarkably constant values both over the control period and during the times of increase in numbers. Only during the terminal phases, at times of overall large increas it was found that some vascularized areas would have phenomenally high numbers (e.g. 7 x the normal count, as opposed to 4x the normal count for the rest of the preparatic These departures from the average are recorded as dotted lines on the graphs.

During the course of these mast cell counts it was possible to make a detailed examination of the tissues and to note the appearance of plasma cells, lymphocytes, eosinophils and neutrophils, phagocytosis and all the other manifestations of cellular damage and change noted in this thesis.

Haematological methods.

Absolute leucocyte and platelet counts.

The procedure was based on that of Rees and Ecker (1923) for platelet counts and was carried out as follows.

(a) Sodium Citrate 1·0 grams.
Mercuric Chloride 0·002 grams.
Brilliant Cresyl Blue 0·02 grams.
dissolved in 100 mls. of distilled water.

(b) Urea 20 grams, dissolved in 100 mls. distilled water.

The diluting fluid was prepared immediately before use by mixing equal volumes of (a) and (b); the mixed solutions were then filtered through fine filter paper.

Approximately 20 mls. of blood were taken from the jugular vein of the animal into a polythene cup, and a sampl diluted immediately, 1:20 with the diluting fluid using a Thoma pipette. The blood was sandwiched between diluting fluid in the measuring part of the pipette before filling completely by withdrawing into the diluting chamber. The pipette was then shaken for at least two minutes to mix thoroughly, and care was taken throughout to avoid contamina with dust particles.

To make the counts a Neubauer haemocytometer was used. The diluting pipette was shaken mechanically for 2 minutes and then about half of the contents in the mixing chamber expelled; each side of the haemocytometer was then filled with a single drop from the pipette. If the haemocytometer was absolutely free from grease and the filling performed smoothly and rapidly the distribution of the cells was very even. After filling, the haemocytometers were placed in Petri dishes containing moistened cotton wool for fifteen minutes to allow the platelets to settle.

The leucocytes were counted on low power magnification on four outer 1 sq.mm. areas each side of the chamber. The sum of the eight values divided by 40, expresses the leucocytes present in the whole blood in thousands / cu.mm. If the eight values varied widely, more areas were counted and the inconsistant values rejected.

Platelets were counted in the small squares in the central areas of the crosses on both sides of the haemocytometer at higher magnification. The sum of five groups of sixteen squares gave a value equal to the platelet numbers of whole blood in thousands / cu.mm.

The peculiar refractive properties of platelets

were used to distinguish them from the cell debris and occasional dust particles.

Erythrocyte Count.

These were made on oxalated blood (potassium oxalate 3mg./ml.); a dilution of 1:200 was carried out in a Thoma pipette and the counts made in a haemocytometer.

Differential Leucocyte Counts.

A thin smear of blood was made, from a drop taken up by a platinum loop from the polythene cup, on a microscope slide by means of a cover slip. It was vital to clean these slides with Chromic acid, hot water, distilled water and ethanol in turn, to ensure that no grease was present. The smears were dried quickly over a stream of warm air if necessary and stored in a dry cupboard for at least twenty four hours.

The slides were then stained with Wright's, diluted 1:2 while in contact with the smear; for 10-15 minutes on a slide rack.

Each slide was then washed quickly with distilled water and differentiated by immersion in 0·08% acetic acid for 5-7 seconds, blotted well and allowed to dry. A total of 5000 cells were counted, using two different preparations as a check.

# EXPERIMENTAL.

The first animals available at the beginning of this work were three calves of approximately 125 Kg. in weight, which were to be used to test the activity of bracken frond (Humphreys D.J. 1963). No comparative estimate can be given as to the amount of toxic substance each animal received since neither the amount originally extracted from the fresh frond nor the amount subsequently lost during the extraction procedure was known. However the three calves were fed the extract fractions at the same rate with respect to the original material. The activity of the original material was not tested, but the dose level used (12 lbs. of fresh frond/ day) is usually sufficient to kill a calf in 30 days, although variation in bracken samples does occur. In all this work the amount of haemopoietic factor was estimated not in terms of weight of material given, but in terms of the effect produced.

The animals received the test fractions by drench as an aqueous suspension in two daily doses in the periods shown, otherwise they received the normal diet. The animal receiving the alcohol precipitate (Calf III) was given the solid material mixed with bran.

Tissue mast cell levels were followed in the three calves by taking serial samples of subcutaneous connective tissue. Total and differential blood counts were performed and the circulatory heparin levels followed on the same samples.

The results are shown in the following pages and figures.

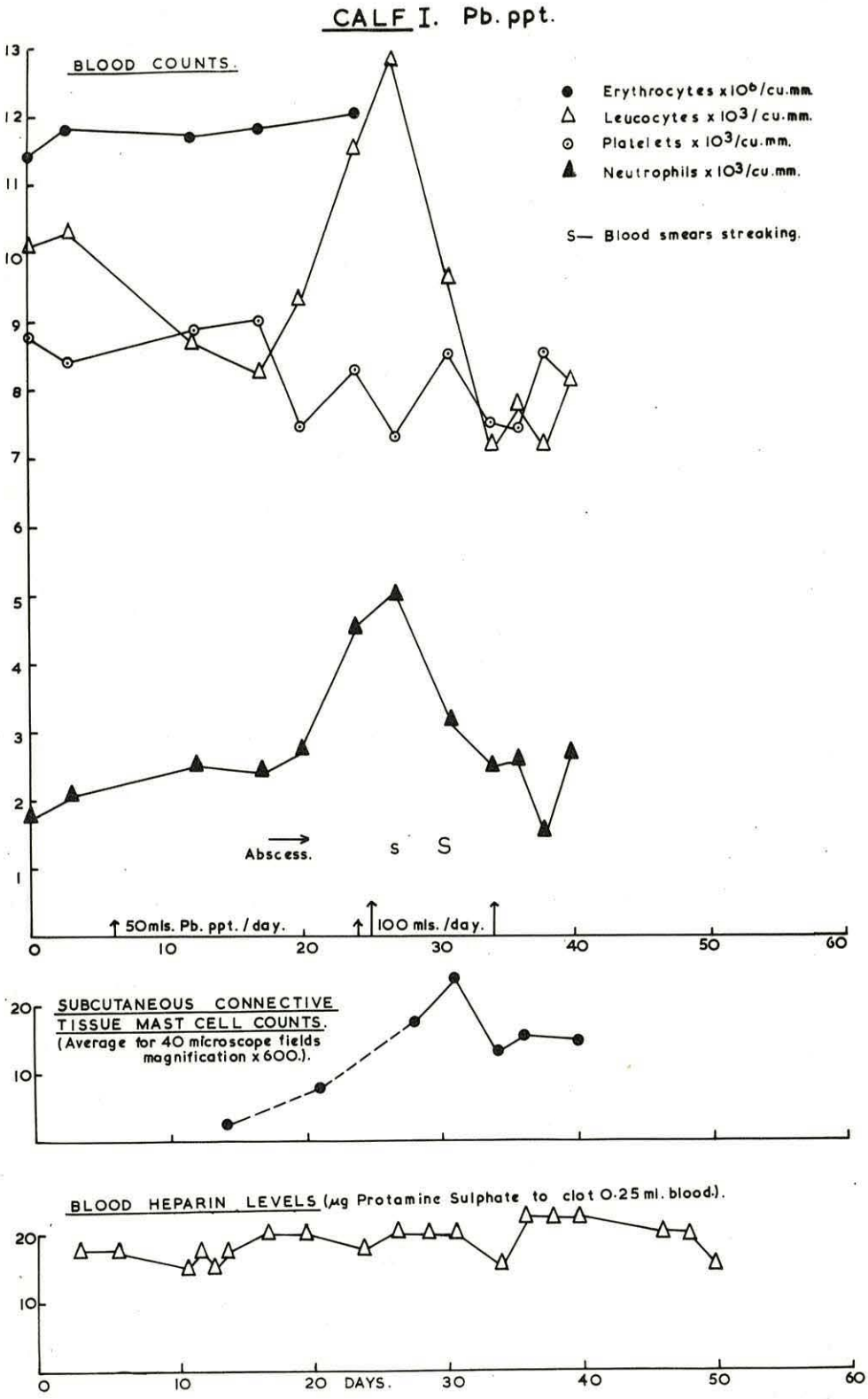

CALF I. Pb. ppt.
BLOOD COUNTS.
Erythrocytes x10⁶/cu.mm.
Leucocytes x 10³/cu.mm.
Platelets x 10³/cu.mm.
Neutrophils x 10³/cu.mm.
S— Blood smears streaking.
Abscess.
s S
50mls. Pb. ppt. / day.
100 mls. /day.
SUBCUTANEOUS CONNECTIVE TISSUE MAST CELL COUNTS.
(Average for 40 microscope fields magnification x 600.).
BLOOD HEPARIN LEVELS (µg Protamine Sulphate to clot 0·25 ml. blood.).
DAYS.

Fig. 3.

Calf I.

Calf I received the lead precipitable material which did not contain any apparent activity, since the blood counts did not show the dramatic 20 day leucocyte and platelet fall characteristic of bracken poisoning. The platelets, usually the most sensitive indicators, remained at a steady level. However, on the 18th day the animal was noticed to be suffering from an abscess associated with an umbilical hernia; this probably accounts for the leucocytosis seen between day twenty and day thirty. The increase was due mainly to the transient neutrophilia shown, although eosinophils normally absent in this calf were present as 1% of the differential count on days 20 and 24. The infection was probably localized since no temperature increase or other signs of a generalized bacteraemia were noted.

The numbers of tissue mast cells in the samples of subcutaneous connective tissue are shown in the figure. Although an apparent peak is visible it should be noted that the initial value is much lower than the normal level, while the highest value (23•7) in the light of subsequent work is only slightly over normal control values. The blood smears prepared for differential counts streaked slightly on day 27 and more noticeably on day 30; no circulatory heparinoid anticoagulant was detected at this period, although these occasions appeared to coincide with the highest mast cell values.

Too few samples were taken to indicate if the mast cell increase was significantly above the normal level for this calf, but any change was almost certainly due to the effect of the infection rather than the bracken extract.

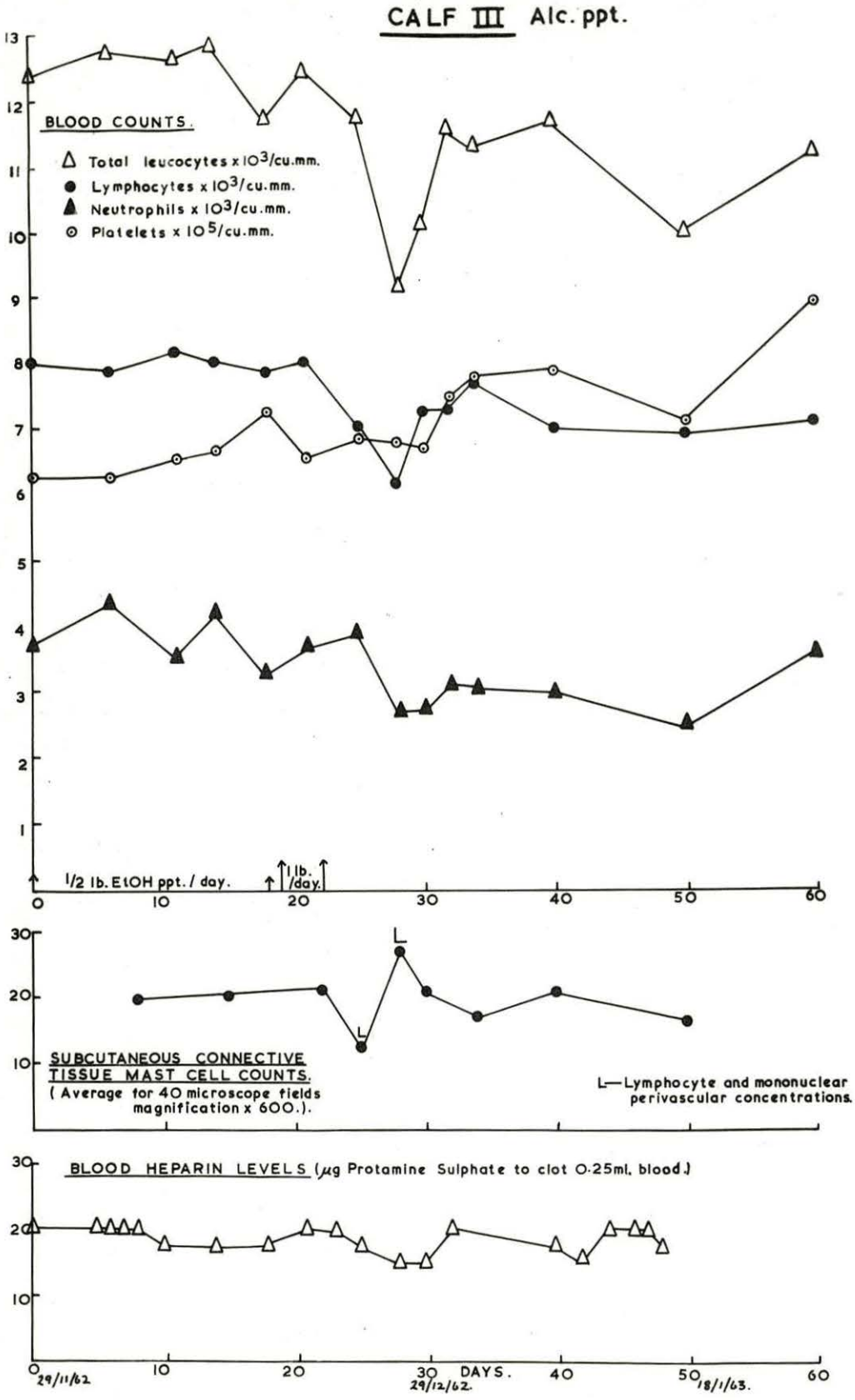

CALF III Alc. ppt.
BLOOD COUNTS.
Total leucocytes x 10³/cu.mm.
Lymphocytes x 10³/cu.mm.
Neutrophils x 10³/cu.mm.
Platelets x 10⁵/cu.mm.
1/2 lb. EtOH ppt. / day.
1 lb. /day.
SUBCUTANEOUS CONNECTIVE TISSUE MAST CELL COUNTS.
( Average for 40 microscope fields magnification x 600.).
L—Lymphocyte and mononuclear perivascular concentrations.
BLOOD HEPARIN LEVELS (μg Protamine Sulphate to clot 0·25ml. blood.)
DAYS.
29/11/62
29/12/62.
18/1/63.

Fig. 4.

Calf III.

Calf III received the material precipitable by ethanol from the original extract; this may have contained a very slight amount of activity since a slight fall in leucocytes (chiefly in neutrophils) occurred from the 25th to the 28th day after the commencement of feeding, however no parallel platelet decrease occurred.

A significant rise (27•4 cells/ field) in mast cell numbers can be seen on day 28 and the preparations from both this day and day 25th contained perivascular concentrati ons of lymphoid type cells characteristic of inflammation. A fall in the circulating lymphocytes was seen on day 28 when this phenomenon was particularly marked. No streaking of the blood smears occurred during the experiment and no heparin release was detected.

It is possible that the tissue reactions seen are a result of the bracken fed, although the circulatory cells did not show dramatic changes.

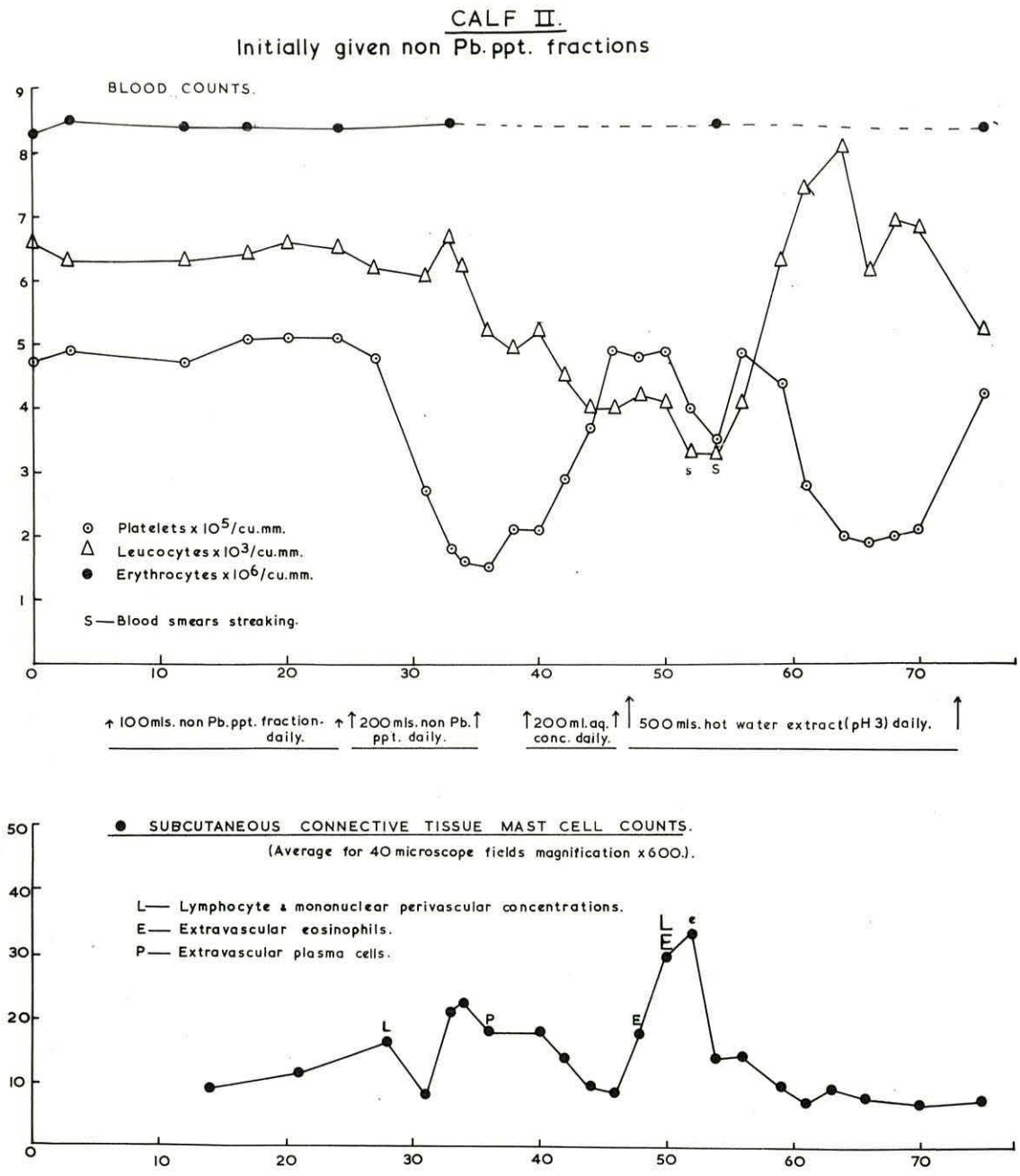

CALF II.
Initially given non Pb. ppt. fractions
BLOOD COUNTS.
Platelets x 10^5/cu.mm.
Leucocytes x 10^3/cu.mm.
Erythrocytes x 10^6/cu.mm.
S — Blood smears streaking.
s
S
100 mls. non Pb. ppt. fraction. daily.
200 mls. non Pb. ppt. daily.
200 ml. aq. conc. daily.
500 mls. hot water extract (pH 3) daily.
SUBCUTANEOUS CONNECTIVE TISSUE MAST CELL COUNTS.
(Average for 40 microscope fields magnification x 600.).
L — Lymphocyte & mononuclear perivascular concentrations.
E — Extravascular eosinophils.
P — Extravascular plasma cells.
L
P
E
L
E
e

Fig. 5.

Calf II.

Calf II received initially the material not precipitable by lead (Humphreys D.J. 1963) in the period and amounts shown. This fraction contained the main activity since a significant change in the blood counts was produced. The platelets in particular showed a marked decrease. It was decided to attempt to induce the fatal syndrome in this calf using a simple extract, so the test fraction was followed by an aqueous concentrate prepared in the manner of Evans et al. (1959).

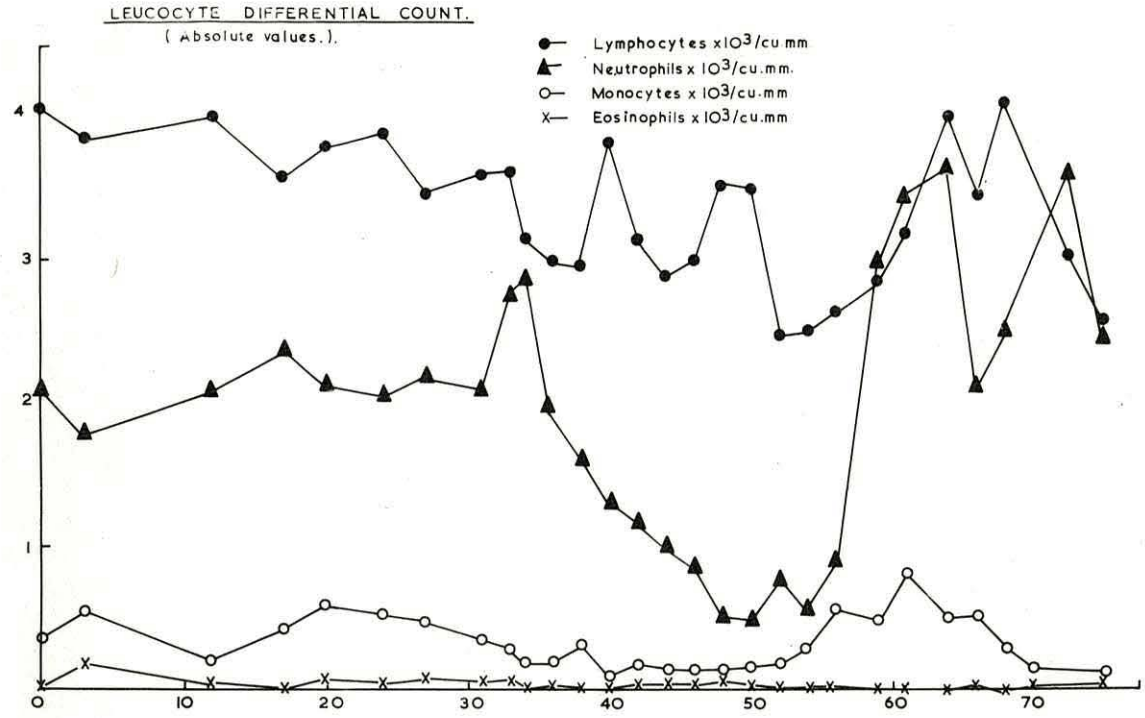
Fresh frond was minced into water at boiling point, the mixture was adjusted to pH 3 with concentrated acetic acid, allowed to cool, and filtered through muslin. The filtrate was then concentrated until 500 mls. was equivalent to 12·5 lb. of fresh bracken frond.

This was adjusted to pH 6 with NaOH before being given by drench to the calf, and 200 mls. of this (later increased to 500 mls.) was given to the calf on the dates shown, after the termination of the test fraction dosage.

It can be seen from the graph that this caused the platelets, which had returned to a normal level, to fall again, while the leucocytes continued to fall. Smears prepared for differential counts were observed to streak slightly on day 52 and more strongly on day 54, this coincided with a slight appearance of circulating heparinoid anticoagulant on day 54.

A very noticeable increase in mast cell numbers was apparent on days 50 and 52 when the leucocytes were at their lowest, associated with a tissue eosinophilia. Eosinophils are not usually seen in connective tissue in the normal bovine, although they are considered to be a

## CALF II.


LEUCOCYTE DIFFERENTIAL COUNT.
( Absolute values.).
Lymphocytes x10³/cu mm
Neutrophils x 10³/cu.mm.
Monocytes x 10³/cu.mm
Eosinophils x 10³/cu.mm
4
3
2
1
0
10
20
30
40
50
60
70

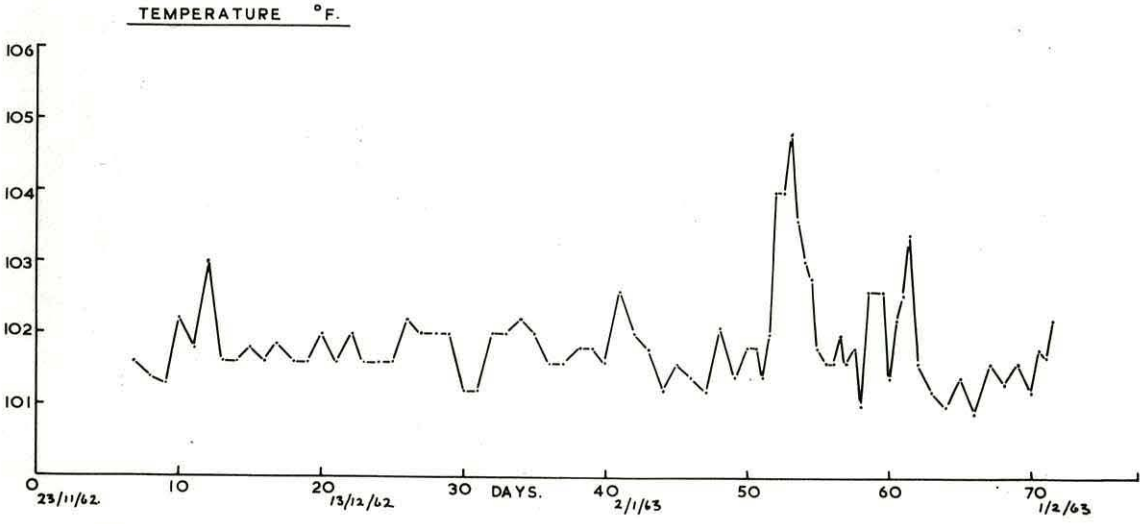

TEMPERATURE °F.
106
105
104
103
102
101
0
23/11/62.
10
20
13/12/62.
30
DAYS.
40
2/1/63
50
60
70
1/2/63

Fig. 6.

normal feature in most mammalian connective tissue. These observations would seem to confirm that mast cell increases, heparin release and streaking were all interrelated phenomena. Apart from a small peak occurring at the period of most marked thrombocytopenia the tissue mast cell levels were very steady at around 10 cells/ field.

The leucocyte differential counts seen in the figure show that the main reason for the leucocyte drop was a marked decrease in the numbers of neutrophils. The monocytes, always a sensitive indicator of bracken injury also show changes, although a marked fall would not affect the total count greatly since these cells comprise only about 10% or lower of the normal bovine leucocytes. A marked fall in the lymphocytes on day 50 is possibly associated with the appearance of the perivascular lymphocyte concentrations in the tissues at the same time as the mast cell increase.

A moderate pyrexia comparable to infections, but not as high as that observed in some bracken cases, was associated with the mast cell increase and the heparin release may have contributed to the pyrexia (Landy and Shear 1957).

In summary it can be said that although none of the animals died, the findings of Evans I.A. and Howell were confirmed, showing that fluctuations in the mast cell population have a significant role in the pathology of bracken poisoning and possibly also in systemic reactions to an apparently local infection. It is perhaps indicative of a beneficial role, that the blood counts in calves II and III rose after the mast cell increases. It was also apparent that more frequent sampling would have to be undertaken in order to obtain a true picture, since the

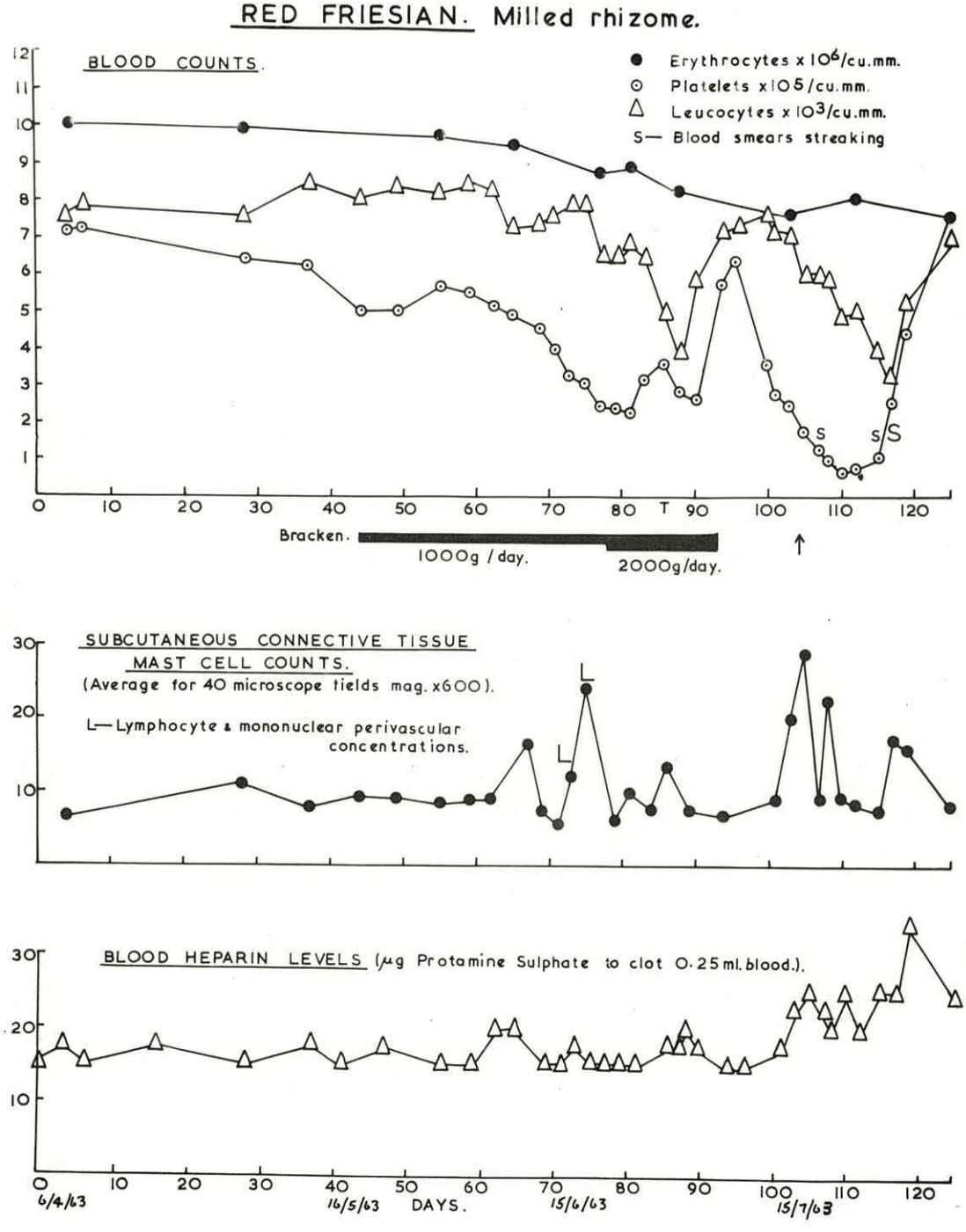

RED FRIESIAN. Milled rhizome.
BLOOD COUNTS.
Erythrocytes x 10⁶/cu.mm.
Platelets x 10⁵/cu.mm.
Leucocytes x 10³/cu.mm.
S— Blood smears streaking
Bracken.
1000g / day.
2000g/day.
SUBCUTANEOUS CONNECTIVE TISSUE
MAST CELL COUNTS.
(Average for 40 microscope fields mag. x600).
L— Lymphocyte & mononuclear perivascular concentrations.
BLOOD HEPARIN LEVELS (μg Protamine Sulphate to clot 0.25 ml. blood.).
6/4/63
16/5/63
DAYS.
15/6/63
15/7/63

Fig. 7.

changes in number took place rapidly and could easily be missed. The tissue samples also revealed evidence of systemic reactions, such as the tissue eosinophilia occurring during the period of mast cell increase in Calf II. Possibly this countered the harmful effects of histamine and other inflammatory agents (Archer and Broome 1963).

No evidence of massive, or petechial haemorrhages was seen in any of the three calves.

Red Friesian.

It was decided to attempt to produce the fatal syndrome using unprocessed bracken since the activity of extracts was variable. At that time of year (February) no fresh frond was available but bracken rhizome seemed to be a satisfactory substitute, especially since Evans et al. (1961) showed that rhizomes, also gathered in February, were at least 5 times as active as the frond. 25, half pound doses of the dried material was a lethal amount.

The rhizome was collected from a steep roadside site in the Nant Ffrancon valley near Bethesda, by removing the overlying soil with a pick and gathering the exposed rhizome by hand. This material was washed to remove soil and air dried at 40°C for 72 hours in an oven, it was then milled and fed as a coarse powder.

The Red Friesian calf was fed initially at a rate of 1000g./day, this was later doubled in an attempt to produce the fatal syndrome. It appeared that the initial dose rate was too low, since the platelets were falling at a relatively slow rate, indicating that the bone marrow was at least partly functional. As can be seen from the figure the increase in dosage appeared to temporarily arrest

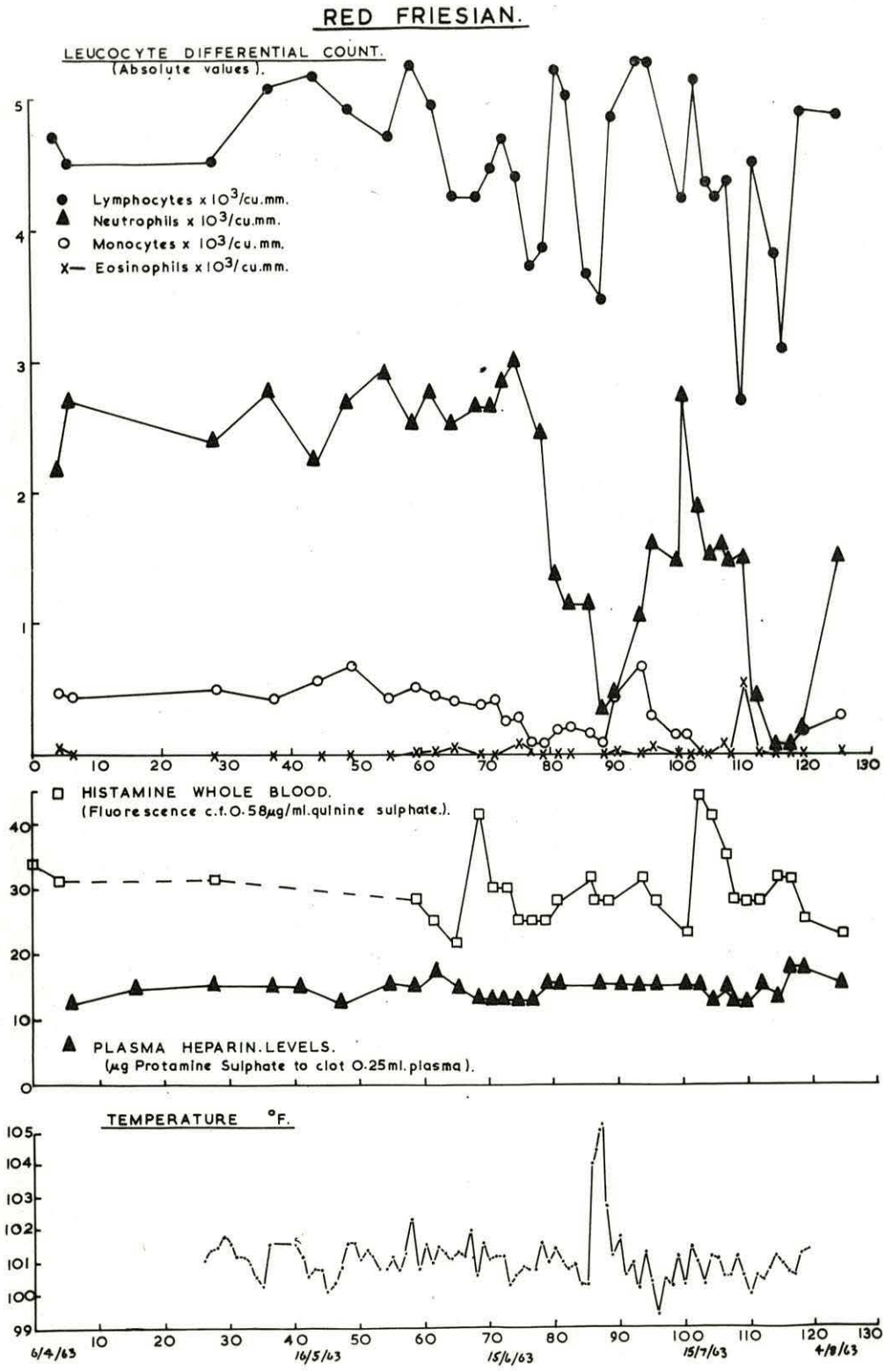

RED FRIESIAN.
LEUCOCYTE DIFFERENTIAL COUNT.
(Absolute values).
Lymphocytes x 10³/cu.mm.
Neutrophils x 10³/cu.mm.
Monocytes x 10³/cu.mm.
Eosinophils x 10³/cu.mm.
HISTAMINE WHOLE BLOOD.
(Fluorescence c.f. 0·58µg/ml. quinine sulphate.).
PLASMA HEPARIN. LEVELS.
(µg Protamine Sulphate to clot 0·25ml. plasma).
TEMPERATURE °F.
6/4/63
16/5/63
15/6/63
15/7/63
4/8/63

Fig. 8.

the rise in platelet levels, starting as the dose was increas and also to cause a sharp leucocyte fall.

Dosage was discontinued when the supply was nearly exhausted and it had become obvious that the blood counts were increasing rapidly, the remainder of the rhizome powder (1800 gm.) was given as a single dose on day 104.

It can be seen from the figure that after the initial rise in the blood cells following the dose increase, a second more marked decrease occurred. During the period of most marked thrombocytopenia, circulatory heparin was associated with increases of the tissue mast cells to levels significantly over the very steady normal numbers. It can be seen however, that not all the mast cell increases were followed by the appearance of circulatory heparin, although all the mast cell increases are associated with periods of "crisis" at the time of the blood cell falls.

The fall in leucocyte numbers was due mainly to the marked reduction in neutrophils (Figure 8), but the level of monocytes also decreased. Two slight peaks in the whole blood histamine content appear to be coincident with the marked increases in mast cells. No increase was detected at the time of the large circulatory heparinoid release around day 120; however, since whole blood and not the plasma or free histamine was measured it is possible that the important changes in functionally active histamine may have been masked by fluctuations in the blood cells or by the amounts in the blood cells (histamine in mast cells for example, can be released without cell disruption). The plasma histamine was not measured at this stage as it was feared that the continuous serial blood removal would affect the animal. An attempt was made to keep the volume removed to a minimum, however it was realized that increases in

functional histamine could not be detected with certainty unless plasma determinations were carried out in parallel. The histamine did not appear to follow the blood basophil levels since these were very low throughout and the slight changes seen were not related to the histamine fluctuations.

The mast cells on day 110 (following a small peak on day 108) were observed to be breaking up. The marked circulatory eosinophilia evident on this day (Figure 8) was possibly related to this and it can be seen that streaking also appeared at this point.

No histamine increase was visible, possibly had the animal been able to muster an eosinophil response this would have been evident and the animal exposed to the harmful effects. Wells (1962) found that the eosinophil response was greater in rats which surviving parasite infestations, developed strong immunity, and she supported the idea of eosinophil-histamine antagonism. A short period of pyrexia was observed at the time of greatest neutropenia but this did not appear to be associated with a mast cell increase, widespan antibiotic was given automatically during the period of high temperature for this and subsequent calves.

No haemorrhages or loss of condition was visible and after the termination of the experiment the animal has remained apparently normal.

This experiment illustrates the main difficulties experienced in this work, i.e. the time and labour involved in collecting the large amounts of bracken required, and the variations in potency of the material.

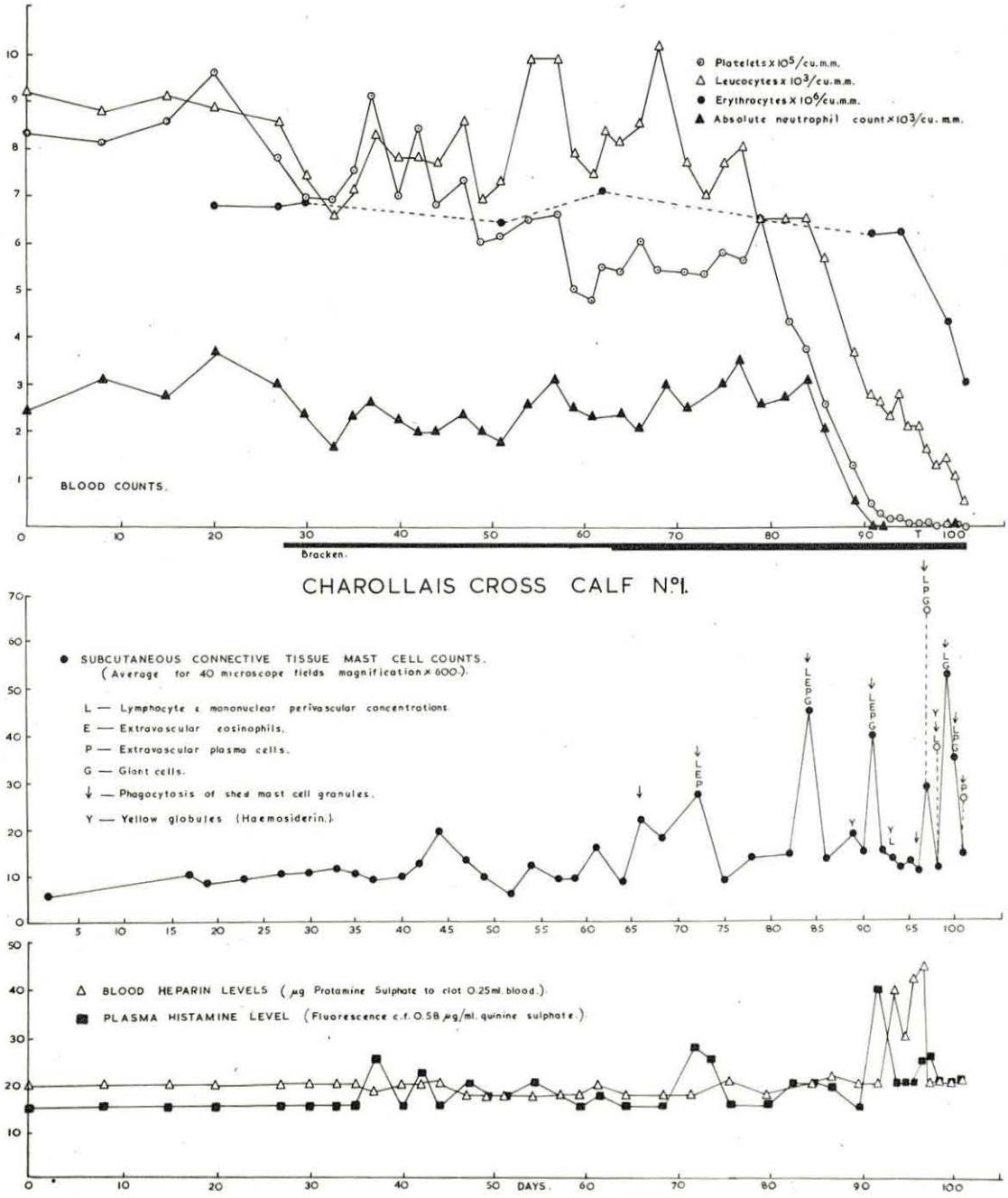

Platelets x 10^5/cu.m.m.
Leucocytes x 10^3/cu.m.m.
Erythrocytes x 10^6/cu.m.m.
Absolute neutrophil count x 10^3/cu. m.m.
BLOOD COUNTS.
Bracken.
CHAROLLAIS CROSS CALF N°1.
SUBCUTANEOUS CONNECTIVE TISSUE MAST CELL COUNTS.
(Average for 40 microscope fields magnification x 600.)
L — Lymphocyte & mononuclear perivascular concentrations.
E — Extravascular eosinophils.
P — Extravascular plasma cells.
G — Giant cells.
↓ — Phagocytosis of shed mast cell granules.
Y — Yellow globules (Haemosiderin.)
BLOOD HEPARIN LEVELS (µg Protamine Sulphate to clot 0.25ml. blood.)
PLASMA HISTAMINE LEVEL (Fluorescence c.f. 0.58 µg/ml. quinine sulphate.)
DAYS.

Fig. 9.

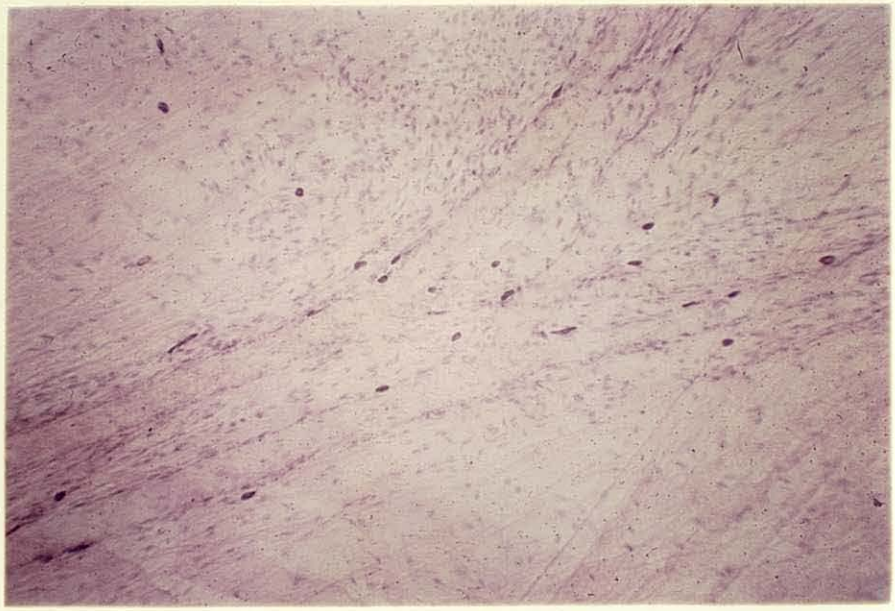
Charollais I.

The detailed results obtained from this calf are shown in the figure.

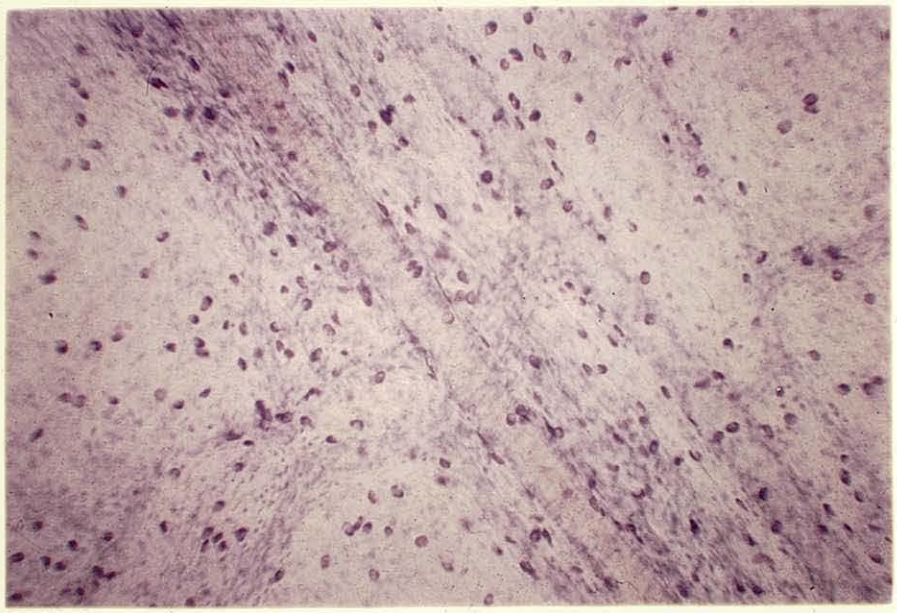
The calf was initially given a freshly prepared hot water extract of frond prepared by the method of Evans et al.(1959). Possibly the original material was inactive as only a slight reduction of platelet levels resulted from the feeding of the equivalent of 10lb./day fresh bracken; This amount of active material has previously been shown to be sufficient to kill a calf in about 30 days. After the exhaustion of this source of material, a dried powder prepared by the method described in Section II, was given at the rate of 1Kg./day dry weight (corresponding to about 5Kg./day of fresh frond). This proved to be highly active as the marked blood falls typical of bracken poisoning started about 20 days after the commencement of feeding, and was followed by a fatal termination after 37 days on the highly active material. Tissue and blood samples were taken as frequently as possible and the figure illustrates the change.

It should be noted that as in the previous calves a mast cell increase was not necessarily followed by the circulatory phenomena, but the figure shows that the peak on day 91 was coincident with a sharp plasma histamine increase and was followed by a blood heparinoid anticoagulant release and streaking of the blood smears on day 93.

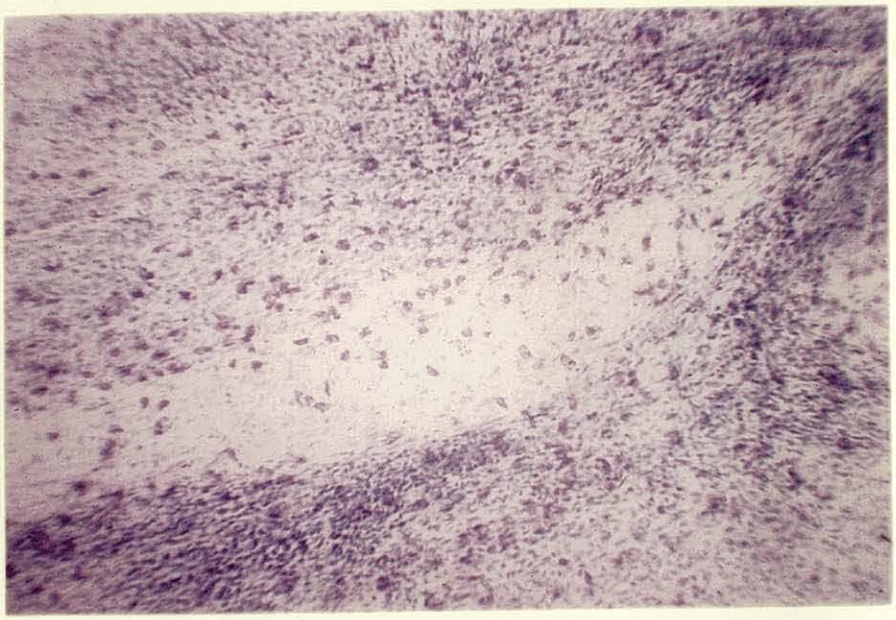
This calf was remarkable in that the tissue samples revealed changes of an intensity and type not previously observed. The mast cell changes were particularly marked and became more frequent and pronounced towards the termination of the experiment. At this phase the mast cell increases although large were more varied, that is, although



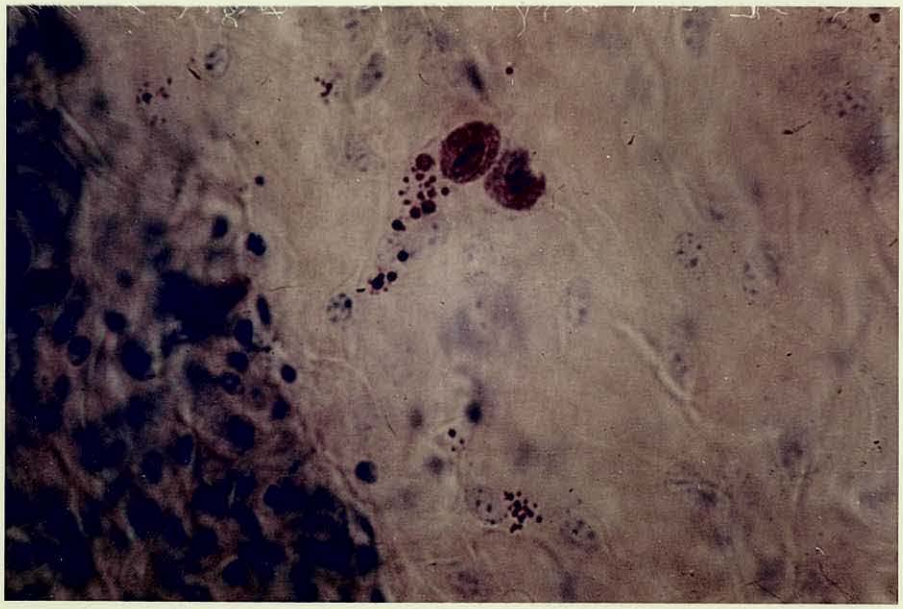
Normal Mast Cell Count. (Charollais I).
Subcutaneous connective tissue (S.C.T.) spread taken as a biopsy sample, x 100, Wright's Stain.



High Mast Cell Count. (Charollais I).
S.C.T. spread, x 100, Wright's Stain.



Very High Mast Cell Count.
(Charollais I). S.C.T. spread, x 100, Wright's Stain.



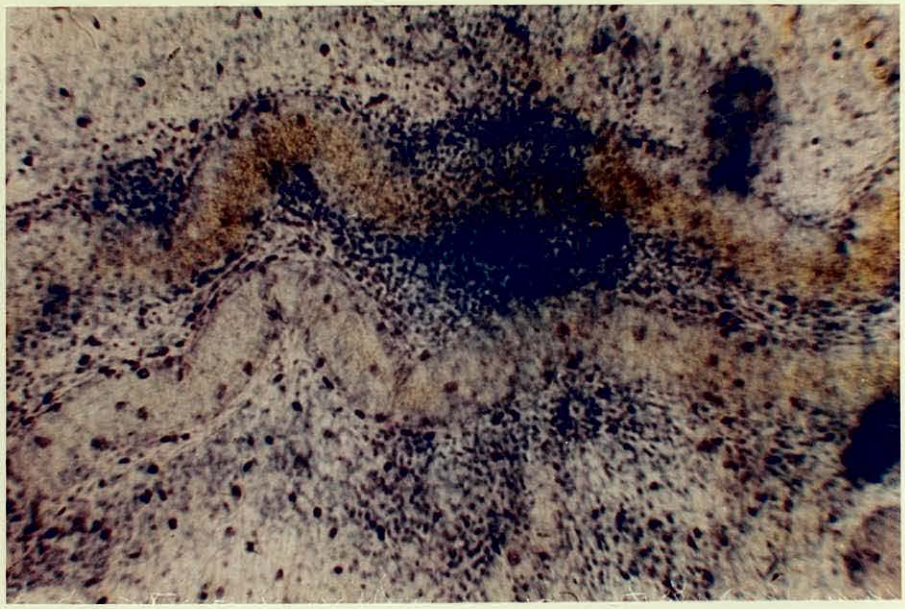
Phagocytosis of Shed Mast Cell granules.
(Charollais I). S.C.T. spread, x 500, Wright's Stain.

the overall levels were increased patches on the preparations contained areas where the counts were very high. The plates illustrate these changes.

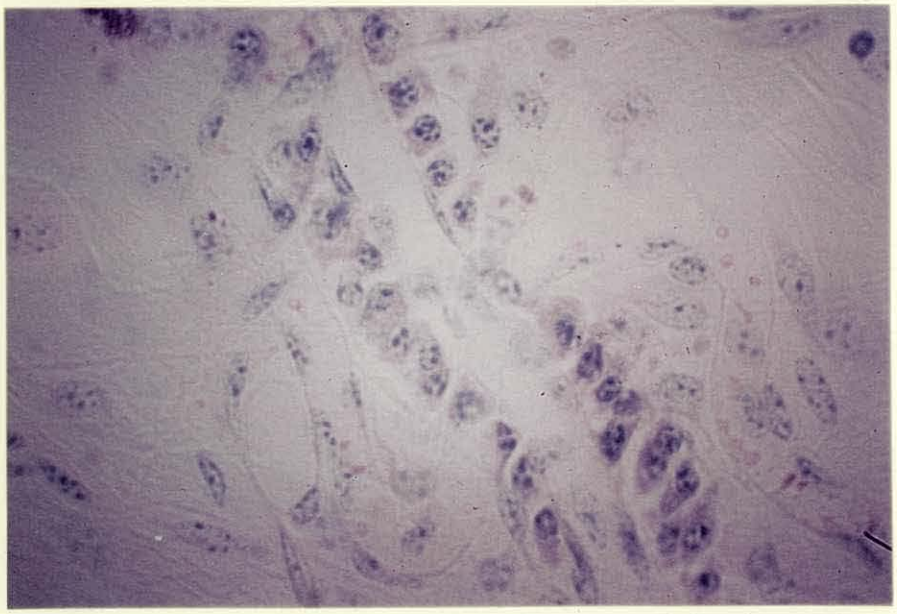
Phagocytosis.

Mast cell granules contained in the cytoplasm of tissue fibroblasts or macrophages could be seen quite clearly on the days of mast cell increases. This is not a normal occurrence (Plate ). The shed granules could easily be differentiated from the scattered granules sometimes seen in the normal tissues, probably an artefact produced by cell disruption by mechanical damage during the fixing and staining processes. The plate shows how the granules appear to coalesce and become more oily in appearance after being shed, even before ingestion.

The mast cell changes were accompanied by other manifestations indicated on the graph and a description is merited at this stage.



Perivascular Lymphocyte and Mononuclear Concentrations.
(Charollais I). S.C.T. spread, x 100, Wright's Stain.

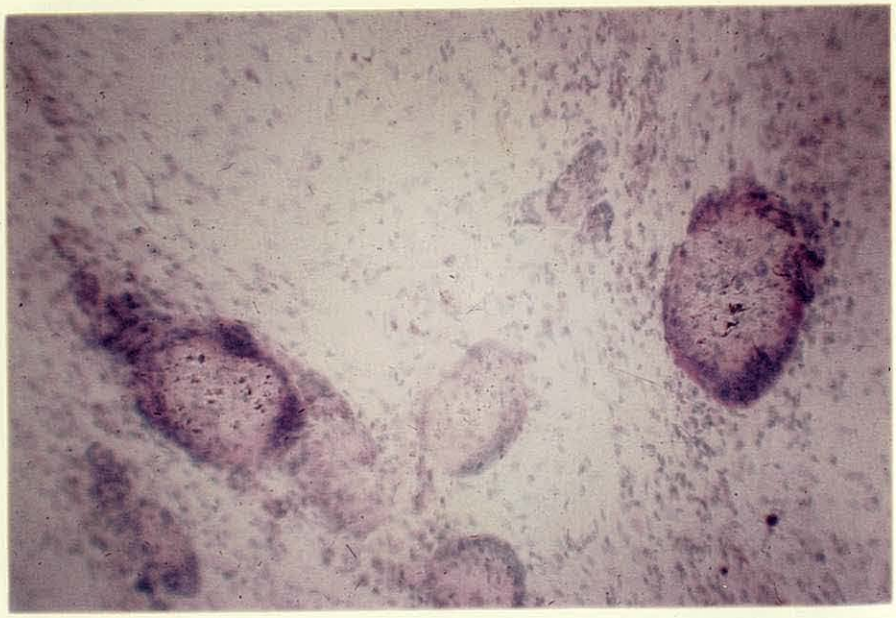


Plasma Cells.
(Calf 152). S.C.T. spread, x 500, Wright's Stain.

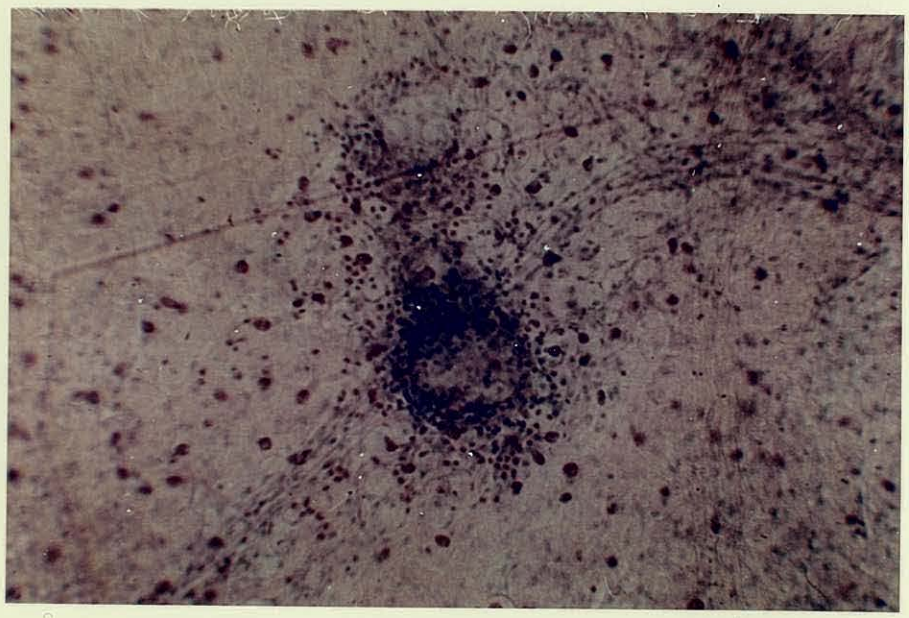
The lymphocytes and mononuclears extravascating from the small blood vessels have been observed in the previous calves although not to the degree seen in this calf (Plate). Thick concentrations of these inflammatory cells could be seen cuffing the blood vessels and scattered less thickly through the surrounding tissues. Many of the cells appeared to be transforming to plasma cells. In the phenomenon there was no preliminary invasion by neutrophils as usually occurs during the normal inflammatory reaction, this is very reminiscent of the delayed hypersensitivity type of reaction where the neutrophil phase is absent or very weak. Although neutrophils were conspicuous by their absence, the eosinophil response varied from mild to frankly massive. In fact, appreciable numbers were found in the tissues even on days when there were no circulating eosinophils.

Plasma cells.

Plasma cells, the main producers of antibodies are partly recognisable by their distinctive staining qualities, the large R.N.A. content consequent upon their high anabolic activity means that the cytoplasm of these cells has a great affinity for basic stains. The pyronin-methyl green stain in particular is used to distinguish these cells, the pyronin staining the cytoplasm pink while the nucleus picks up the methyl green. These cells are also recognisable with Wright's stain (Plate) the cytoplasm staining a mauve colour. As well as their distinctive staining these cells have a characteristic morphology. Cells at all stages of maturation were visible from the early transforming type to the pear shaped mature forms with their characteristic perinuclear space.



Giant Cells in Various Stages of Development.
(Charollais I). S.C.T. spread, x 100, Pyronin Methyl Green.



Lymphocytes and Eosinophils gathering round a small Giant Cell. (Charollais I). S.C.T. spread , x 100, Wright's Stain.

Giant cells.

The most striking feature of this calf was the appearance of giant or multinucleate cells in very large numbers. The cells apparently form by the coalescence of individual cells and the nuclei can be seen congregated around the periphery while the centre contains the pooled cytoplasm. These cells are apparently highly functional since they take up the pyronin stain very strongly, with a clearer area in the centre which may coincide with the pooled Golgi apparatus. All sizes were seen from small clusters of individual cells 40-50μ across, to larger ones 200μ across and the central areas of some of those larger cells often seem to contain a deposit. Sometimes the centra area seems to contain a pattern of radiating, centrifugal canals, which might suggest a secretory function. Furthermore, later study of these mass cells suggests that the slightly basophilic mononuclears which aggregate to form them may at times be found containing a group of clear vacuoles, giving them the appearance of foam cells. The significance of all these features must be followed at some later date.

There seemed no doubt that the appearance of these cells in the connective tissue coincided with periods of inflammatory invasion and mast cell peaks; and as with the other cells, the sequence was very rapid.

It was very interesting to find in quite a number of preparations that there was a very marked congregation of eosinophils around the outside of giant cells, and in areas of active giant cell formation.

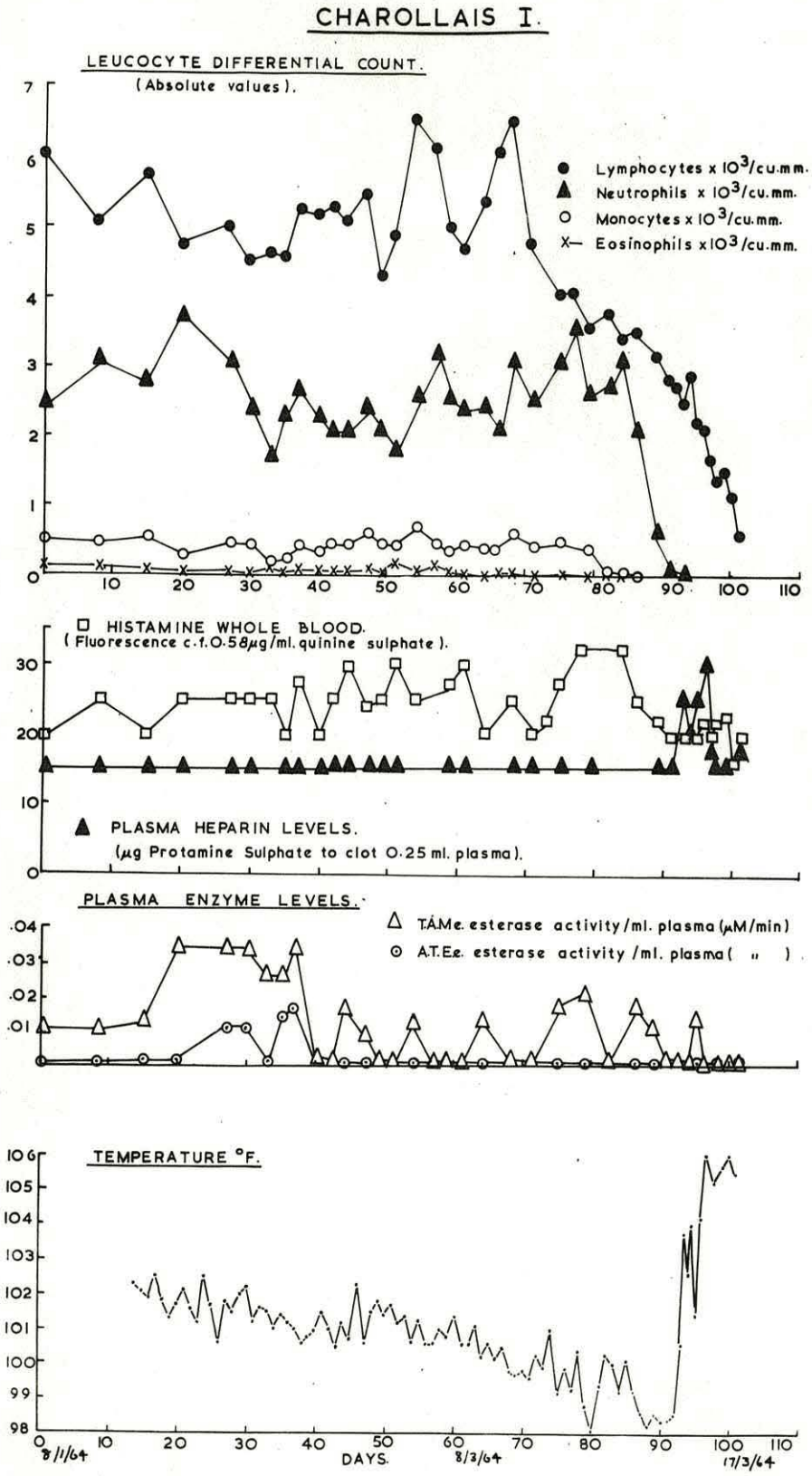

CHAROLLAIS I.
LEUCOCYTE DIFFERENTIAL COUNT.
(Absolute values).
Lymphocytes x 10³/cu.mm.
Neutrophils x 10³/cu.mm.
Monocytes x 10³/cu.mm.
Eosinophils x 10³/cu.mm.
HISTAMINE WHOLE BLOOD.
(Fluorescence c.f. 0.58 μg/ml. quinine sulphate).
PLASMA HEPARIN LEVELS.
(μg Protamine Sulphate to clot 0.25 ml. plasma).
PLASMA ENZYME LEVELS.
T.AMe. esterase activity/ml. plasma (μM/min)
A.T.Ee. esterase activity/ml. plasma ( " )
TEMPERATURE °F.
DAYS.
8/1/64
8/3/64
17/3/64

Fig. 10.

The leucocyte differential counts of this calf show the sharp decrease in neutrophils typical of bracken poisoned calves, while the lymphocytes decrease more gradually.

The plasma heparin level shows a sharp rise at the same time as that shown in the whole blood estimation. The whole blood histamine does not show a rise corresponding to the one in the plasma level. This is perhaps not surprising since histamine once released from the blood cells would be rapidly metabolised.

The plasma enzyme levels remained at very low levels throughout and no massive fibrinolytic increase, which would have been revealed by an increase in T.A.M.e. esterase activity, was shown. However, changes in inhibitor levels, not revealed by the assay, may have been significant and it was decided to attempt to follow these in the next calf. Pyrexia was observed in the final stages at the same time as the heparin and histamine release.

During the last week of life the animal seemed to be suffering considerable intestinal pain, although blood was not observed in the faeces until two days before death. Similarly, although thrombocytopenia was present for a prolonged period during the terminal phase, petechial haemorrhages were not noted until the last few days; possibly the appearance was associated with heparin release. Sterile haematomas also formed at this period in the places where the tissue samples were taken.

However, capillary breakdown may have caused the haemorrhages since patches of necrotic tissue (free from bacteria), were observed in areas of tissue damage on the flanks and also in areas where no apparent damage had been inflicted.

Post mortem examination revealed widespread petechia haemorrhages, in the lungs, heart, intestines and bladder and throughout the loose connective tissue. An intestinal intussusception about 12" in length showed signs of commencing necrosis and a particularly large button ulcer was seen. Gut stasis, possibly as a result of the blockage, appeared to have taken place, and the rumen and proximal intestine were full of material; despite the anorexia shown by the animal during the last week. However, this is reminiscent of irradiated animals in which gut stasis and fluid accumulation occur.

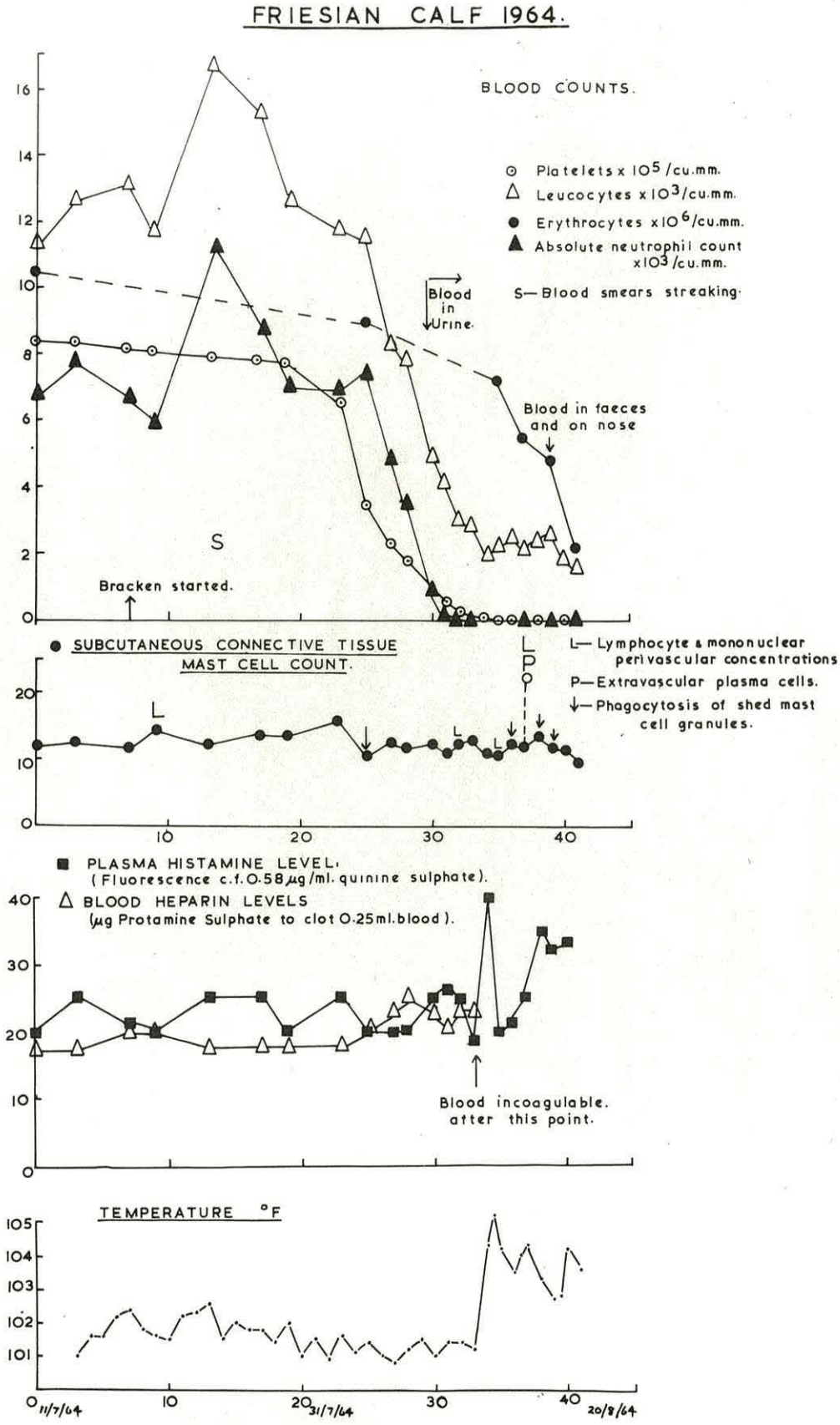

FRIESIAN CALF 1964.
BLOOD COUNTS.
Platelets x 10^5 /cu.mm.
Leucocytes x 10^3 /cu.mm.
Erythrocytes x 10^6 /cu.mm.
Absolute neutrophil count x 10^3 /cu.mm.
S— Blood smears streaking.
Blood in Urine.
Blood in faeces and on nose
S
Bracken started.
SUBCUTANEOUS CONNECTIVE TISSUE MAST CELL COUNT.
L— Lymphocyte & mononuclear perivascular concentrations
P— Extravascular plasma cells.
↓— Phagocytosis of shed mast cell granules.
PLASMA HISTAMINE LEVEL. (Fluorescence c.f. 0.58 µg/ml. quinine sulphate).
BLOOD HEPARIN LEVELS (µg Protamine Sulphate to clot 0.25 ml. blood).
Blood incoagulable after this point.
TEMPERATURE °F
11/7/64
31/7/64
20/8/64

Fig. 11.

Friesian Calf 1964.

This calf was fed the June 1964 dried frond at a rate of 1Kg./day, unfortunately the observations were complicated by the fact that the animal suffered from a kidney disease during the course of the experiment. A heavy infection of C.Renale was diagnosed on day 12 of the experiment and despite the use of antibiotics, this persisted throughout and became markedly worse as the experiment progressed.

Although this calf cannot be taken as a typical case of bracken poisoning, it showed the usual features observed previously. A fall in leucocytes, mainly in neutrophils occurred twenty days after the start of feeding. The platelet decline started somewhat earlier. The marked fall in erythrocytes was probably due to the heavy loss of blood via the urine, an event not usually seen in bracken-poisoned calves and almost certainly due to the kidney infection.

The mast cell levels remained remarkably constant apart from an irregular increase observed in a few areas on the preparations on day 37. A large histamine increase was detected on day 34 and the whole blood remained incoagulable beyond this point. A feature which may be due to either an increase in heparin or to the virtual absence of platelets. A terminal rise in histamine was also detected. A marked pyrexia was also evident from day 34, however, the temperature of 105°F is within the range induced by bacterial infections.

The differential counts revealed the usual sharp decline in monocyte numbers following the neutrophils and a marked eosinophilia appeared to begin after the commencement of the bracken feeding, but this may have been due to the

# FRIESIAN CALF 1964.

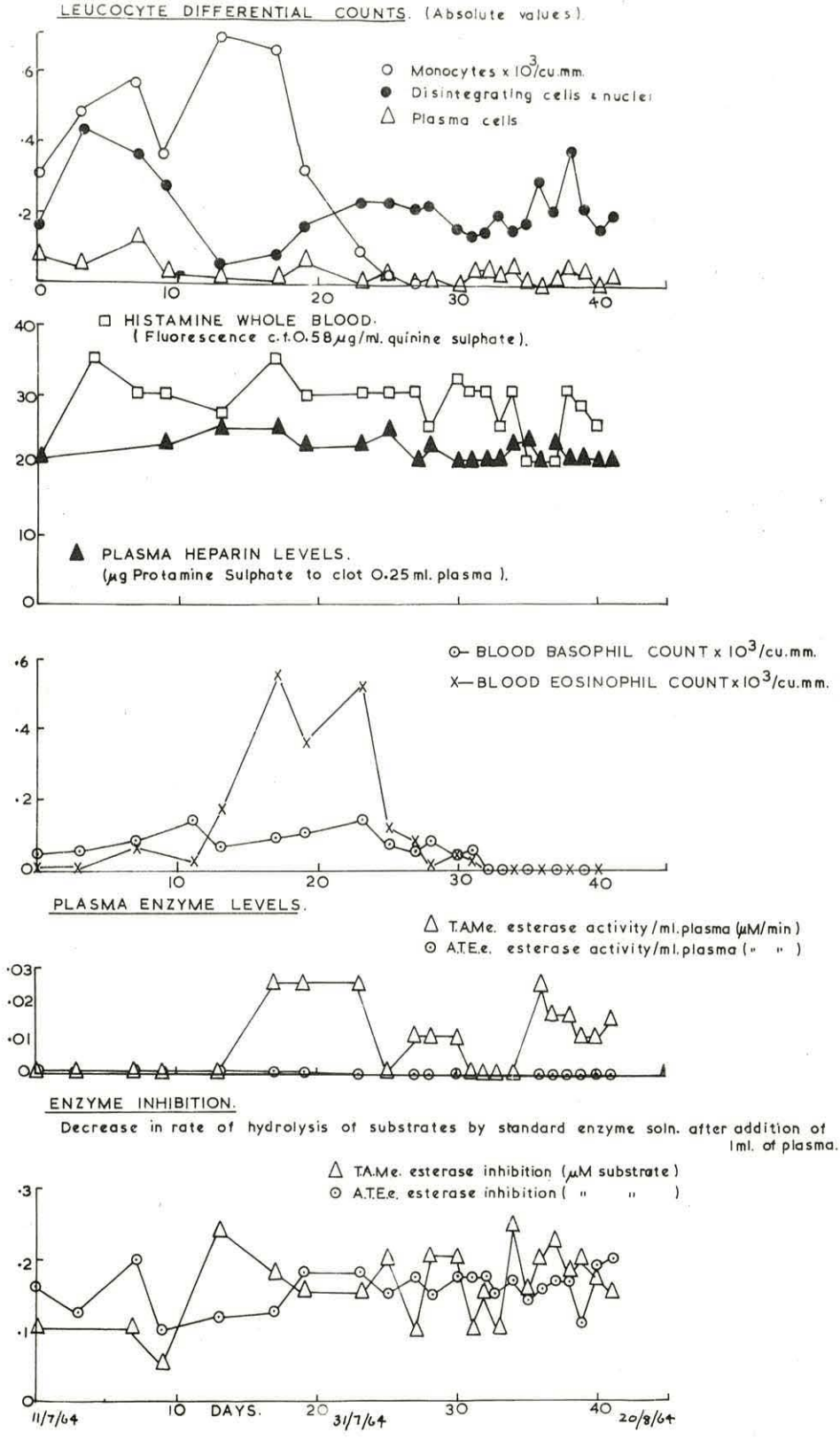



Fig. 12.

infection. The total blood histamine remained fairly steady apart from a possibly significant decrease between days 35 and 37, this may have been due to a discharge of histamine from the blood cells, which could have caused the rise in plasma histamine on day 34. The plasma heparin test failed to reveal any increases in heparin, even when the blood remained incoagulable in the whole blood test.

The plasma enzyme and inhibitor levels did not appear to change significantly during the course of the experiments. Although from the method of plotting, changes may appear to take place, these changes represent a very low level of activity, much lower than would be detected if a release of the kind expected had occurred.

Post mortem examination revealed no large internal haemorrhages although a few small petechiae were visible in the rumen. The kidneys were very degenerate and it is probal that the pyelonephritis due to the Renale infection contribu very significantly to death.

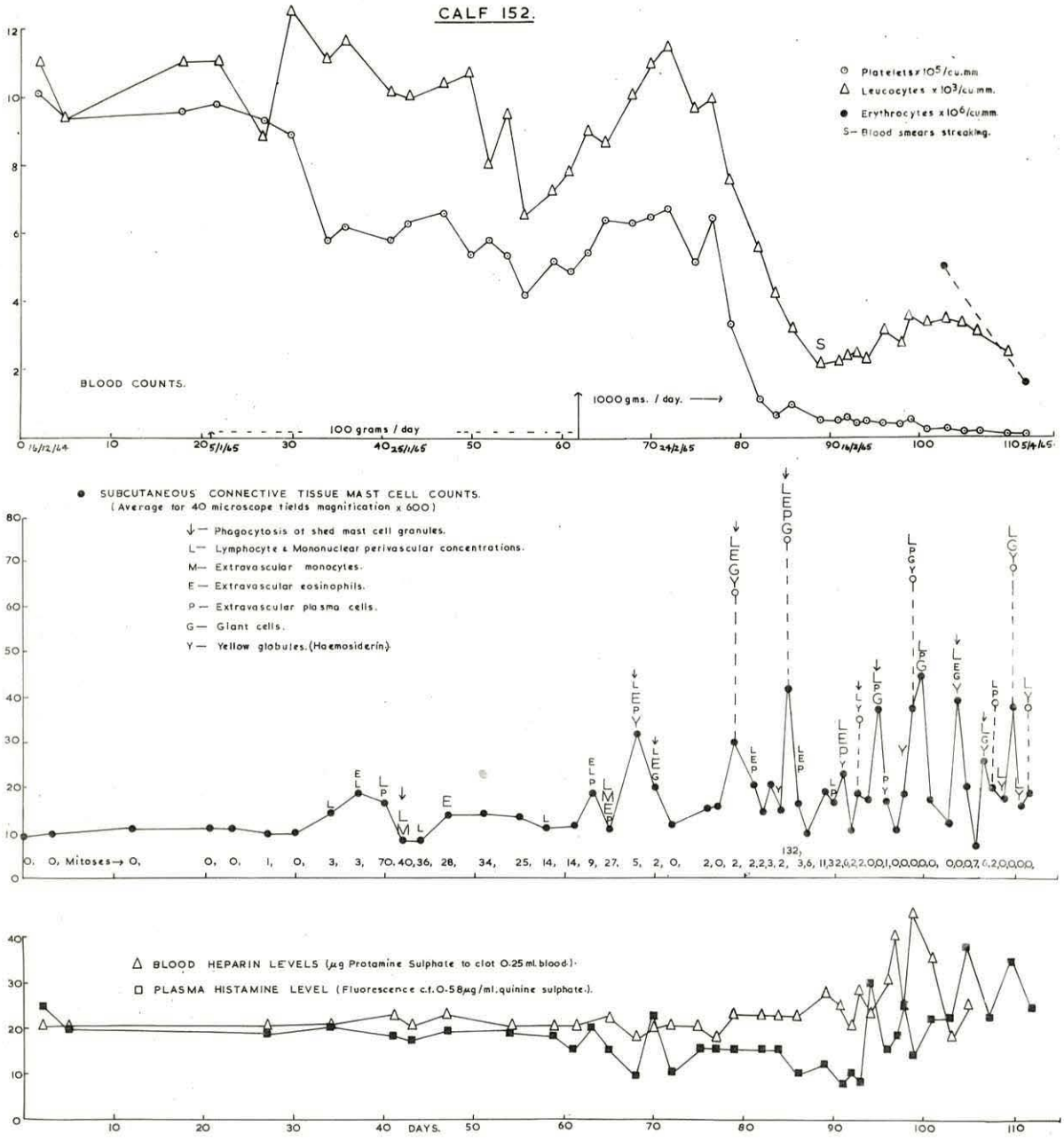

CALF 152.
Platelets x 10^5/cu.mm
Leucocytes x 10^3/cu.mm.
Erythrocytes x 10^6/cu.mm.
S— Blood smears streaking.
BLOOD COUNTS.
1000 gms. / day.
100 grams / day
16/12/44
5/1/45
25/1/45
24/2/45
16/3/45
5/4/45
SUBCUTANEOUS CONNECTIVE TISSUE MAST CELL COUNTS.
(Average for 40 microscope fields magnification x 600)
Phagocytosis of shed mast cell granules.
L— Lymphocyte & Mononuclear perivascular concentrations.
M— Extravascular monocytes.
E— Extravascular eosinophils.
P— Extravascular plasma cells.
G— Giant cells.
Y— Yellow globules. (Haemosiderin).
Mitoses
BLOOD HEPARIN LEVELS (μg Protamine Sulphate to clot 0.25 ml. blood).
PLASMA HISTAMINE LEVEL (Fluorescence c.f. 0.58 μg/ml. quinine sulphate).
DAYS.

Fig. 13.

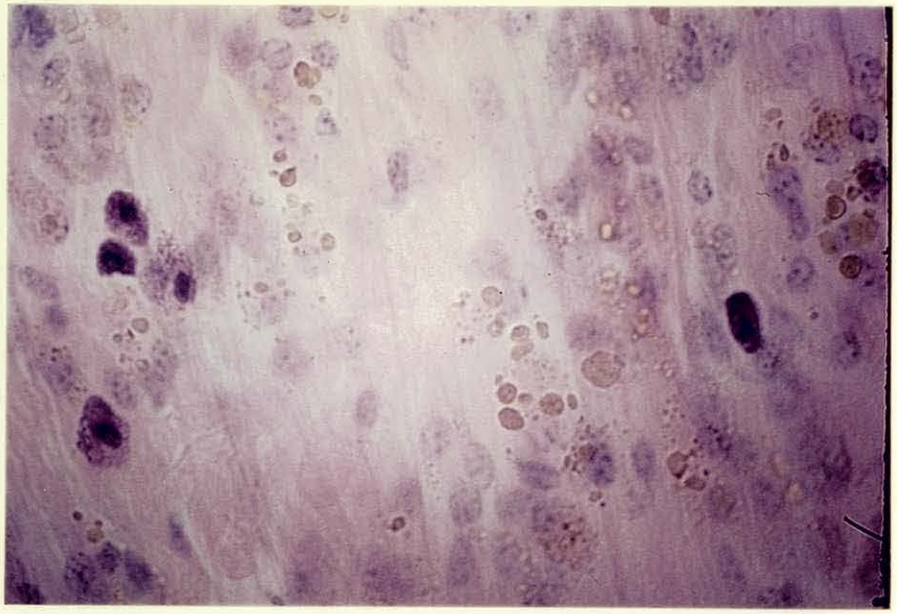
Calf 152.

This calf was one of the most important in the whole series. It was intended to be a repeat of the Charollais I experiment, in which the very marked tissue reactions were observed. The experiment was highly successful in that the results obtained were almost identical as regards both intensity and timing.

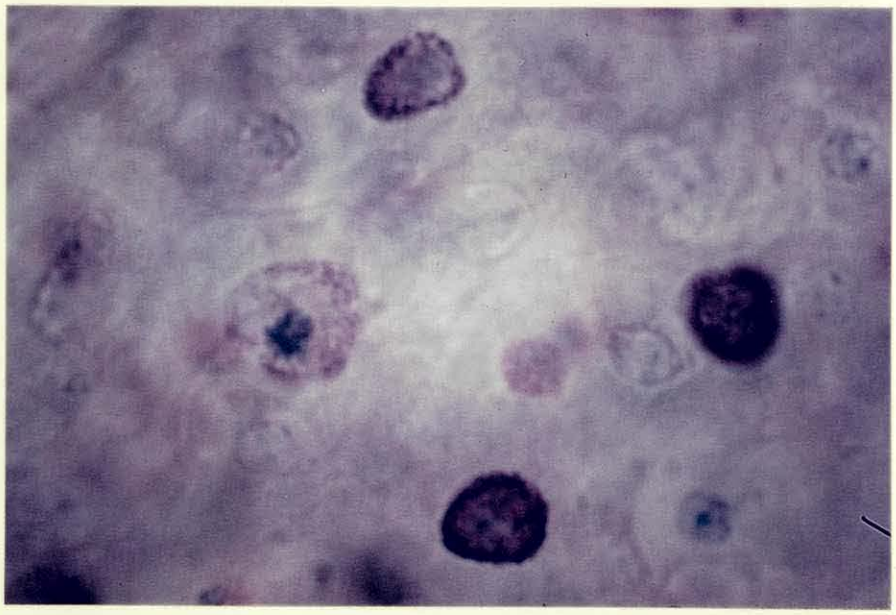
After an initial short control period the calf was given 100 grams/day(to simulate the low activity bracken given to Charollais I) of the June 1964 bracken; after 41 days the amount was increased to 1000grams/ day for the duration of the experiment. This compares with the 35 days of low level given to the Charollais I. A similar slight effect was also observed, in that the platelet numbers of the calf showed an early 40% decrease and remained at that level throughout the period of low dosage. The white cells also showed a gradual decline during this period, unfortunately differential counts were not available at the time of completion of this thesis.

A very sharp decrease in leucocytes and platelets was evident 20 days after the dose increase, after an initial transient increase in leucocytes, but the animal survived for a remarkably long period of time (with widespan antibioti therapy from day 89) with leucopenia and virtual absence of platelets. Possibly the anaemia observed terminally was a major contributor to death.

The tissue manifestations were remarkably similar to the Charollais I, the mast cells in particular showing the same relationship to the doses and blood counts. The same small initial increase of mast cells appeared about 10-15 days after the start of low level feeding, associated



Cells containing yellow globules. (Haemosiderin).
Natural Colour.
(Calf 152). 5/3/65. S.C.T. spread, x 400, Wright's stain.



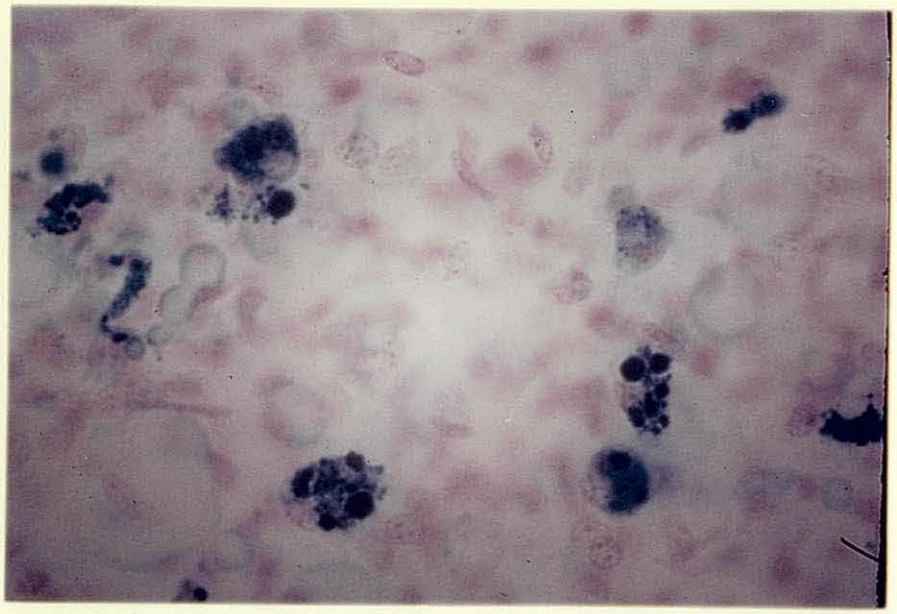
A mast cell in mitosis (Metaphase) with three normal mast cells.
(Calf 152). 11/3/65. S.C.T. spread, x 1000, Wright's stain.

with a similar slight decrease in platelets. An increase was noted a few days after the start of high level feeding, this was also seen in the Charollais I. The very pronounced mast cell increases and tissue reactions started (Fig.13) at the period of circulatory cell decline, as in the case of the Charollais I. The giant, multinucleate cells were in evidence, as prominently as those of Charollais I. The appearance of plasma cells, eosinophils and perivascular lymphocytes and mononuclears was associated with the mast cell fluctuations.

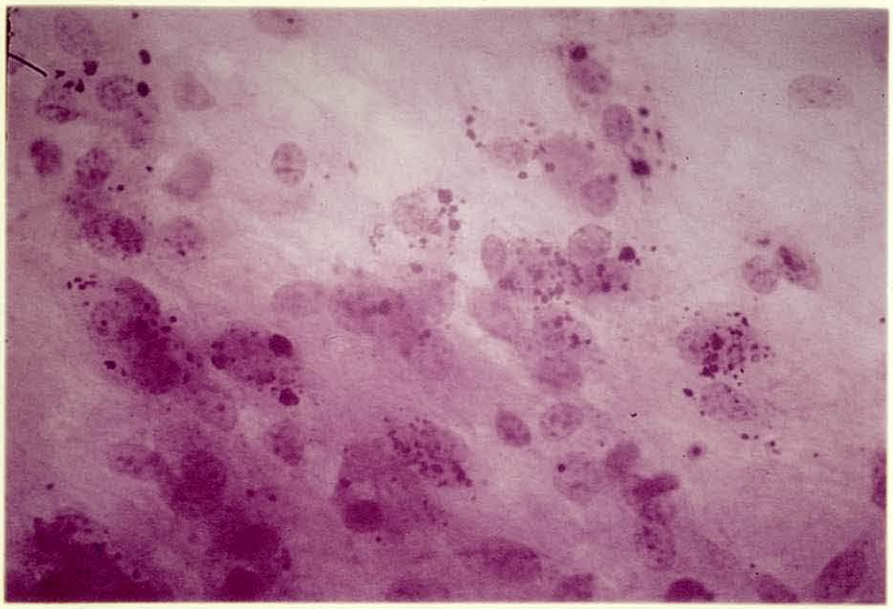
Circulatory heparin and histamine release took place towards the end of the experiment although a small increase in histamine on day 70 may have been associated with the mast cell increase on that day.

Two features not previously noted in other calves were of particular prominence in this calf, these were the appearance of yellow globules and the large incidence of visible mitoses.

The yellow globules occurred particularly at times of the mast cell increases and appeared as oily looking globules in the cytoplasm of the tissue mononuclears. The globules appeared in this form in the normal Wright's preparations and did not pick up either the Wright's or the Pyronin stain. They could even be seen in unstained preparations. The globules almost certainly represented red cells in the process of being broken down, since they were highly reactive with the Quinke stain specific for haemosiderin. The prussian blue stain for iron was also strongly positive. Recognizable red cells were not seen in any of the phagocytes, but it is probable that this mechanism was responsible for a terminal figure of 1·6 million erythrocytes, for no massive haemorrhage was visible, even in the post mortem.



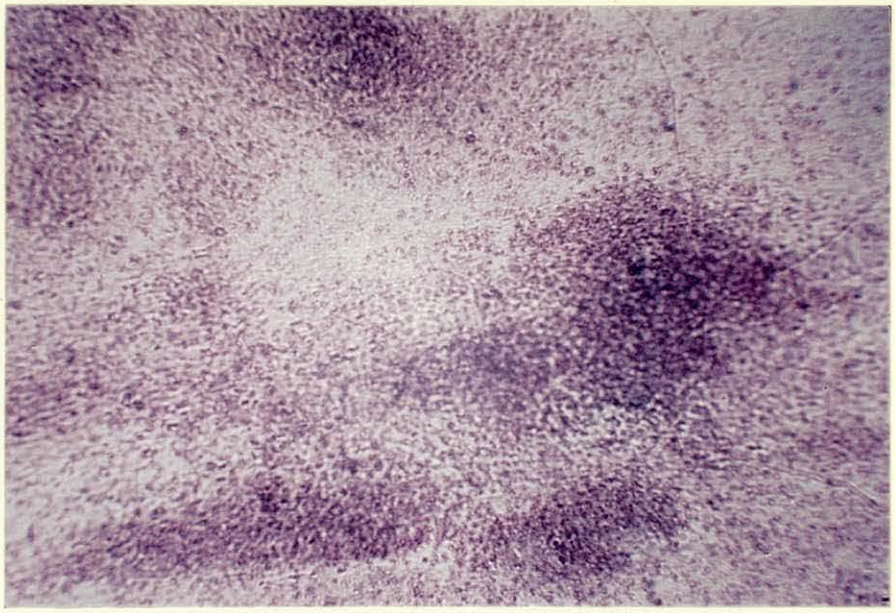
Haemosiderocytes.
(Calf 152). S.C.T. spread, x 400, Prussian Blue stain for Iron.



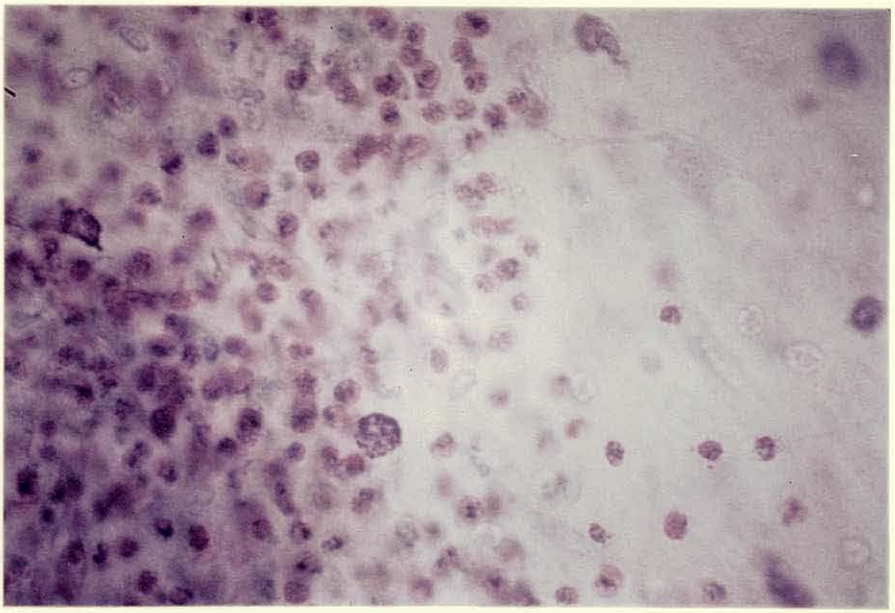
Quincke Reaction for Haemosiderin.
(Calf 152). S.C.T. spread, x 400.

The prominent appearance of mitoses was particularly unusual as these are not normally seen in the tissue samples. Cells at all stages of mitosis were seen, so a Colchicine-like block at a particular phase is unlikely. The cells mainly affected were the capillary endothelial cells and the tissue fibroblasts and mononuclears. 4 mast cell mitoses were visible (on day 85), a very rare event, and on a few occasions mitoses in the plasma cell series were seen, Day 85 in particular was remarkable in both the high numbers of mitoses and in the variety of cell types undergoing mitosis, this must surely be indicative of a systemic stimulant, possibly analogous to the promine of Szent-Gyorgyi et al.(1963). All the mitoses on a preparation normally scanned for the mast cell counts, were noted and these numbers are shown below the mast cell levels in the figure. The observations showed very clear differences, much greater than would be occasioned by the variation in size of the preparations. Two main periods of elevation were observed, one occurring after the first small mast cell increase on day 40 and continuing throughout the period of low dosage, The second tremendous increase occurred just after the blood cell decline, on day 85, and seemed to be associated with the particularly massive increase in mast cell numbers. The meaning of these is not clear, although from the timing of the appearance just after and not before the circulatory cell decline, it would seem that this represents a compensatory systemic increase in mitoses, rather than a retardation.

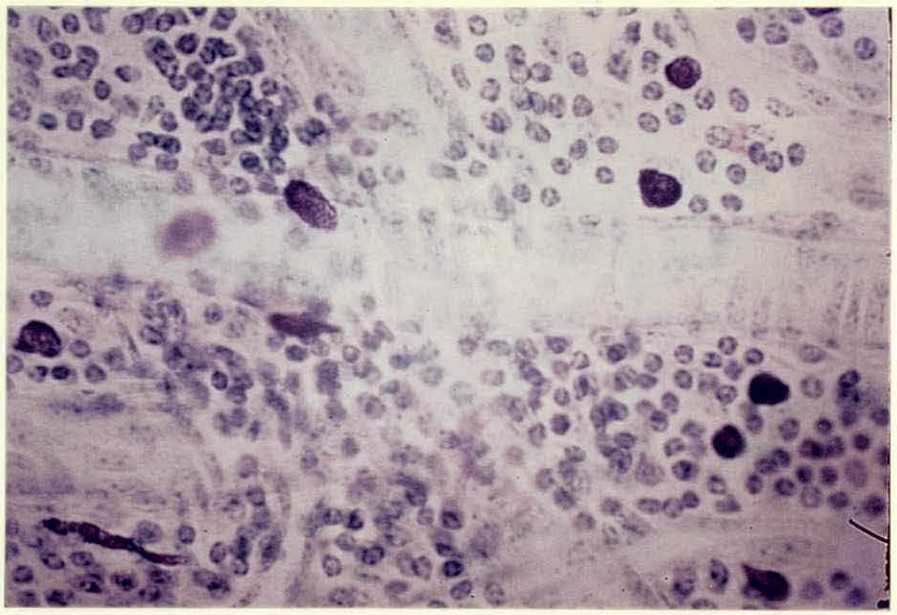
Post mortem examination revealed petechial haemorrhages, although possibly not so pronounced as in other calves. A massive cardiac haemorrhage was the only large one seen and was probably responsible for the sudden death of the animal (before much externally visible distress).



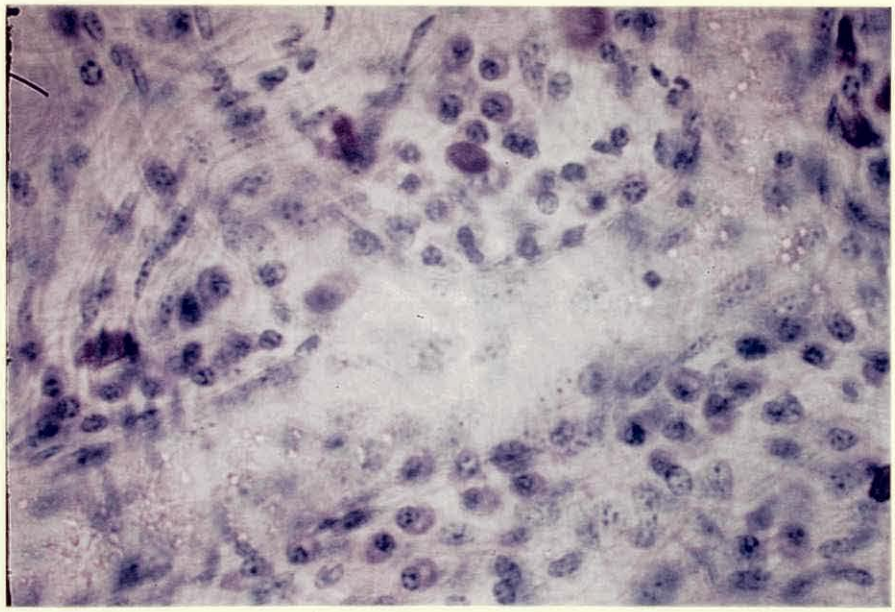
Eosinophils in the tissues in enormous numbers and swarming round giant cells.
(Calf 152). 11/3/65. S.C.T. spread, x 100, Wright's stain.



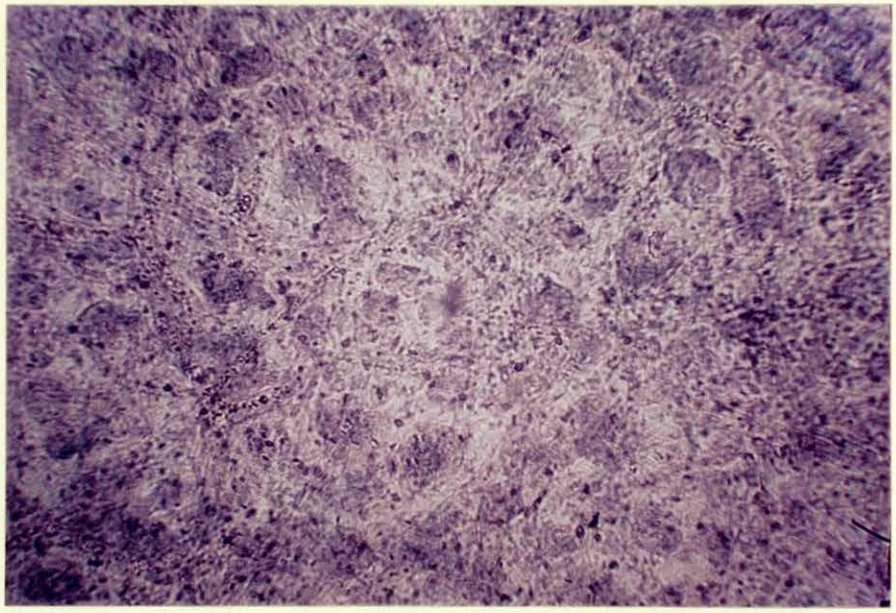
Eosinophils around the edge of a giant cell.
(Calf 152). 11/3/65. S.C.T. spread x 400, Wright's stain.



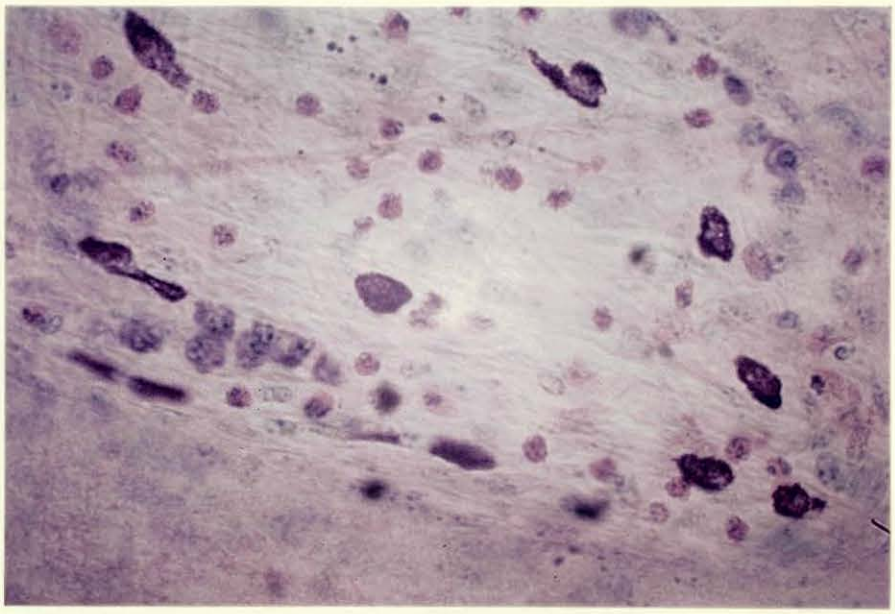
Lymphocytes outside blood vessels, a few beginning to change to plasma cells.
(Calf 152). 30/3/65. S.C.T. spread, x 400, Wright's stain.



Plasma cells outside blood vessels.
(Calf 152). 17/2/65. S.C.T. spread, x 400, Wright's stain.



Giant cells occupying most of the area.
(Calf 152). 21/3/65. S.C.T. spread, x 100, Wright's stain.



Eosinophils and Plasma cells outside blood vessels.
(Calf 152). 17/3/65. S.C.T. spread, x 400, Wright's stain.

# DISCUSSION.

The work has shown that the tissue mast cell levels, while remarkably steady in the normal animal become increased during the course of the bovine bracken poisoning syndrome, especially during the terminal phase. The increases take place rapidly and within 24 hours can increase as much as fourfold and in some localized areas as much as sevenfold. The numbers can drop just as rapidly again to reach the normal levels, so it appears that at least in the tissues studied there is a dynamic sequence of mast cell increases. This is a very different conception from that of a relatively static population, which under certain conditions breaks down and then slowly rebuilds.

The possibility that the biopsy samples are not representative of the animal as a whole must be considered. By taking samples from different areas on the same day during quiescent periods there is no difference between samples; during periods of cellular activity there is more variation, but all the samples on that day would consistently show a raised mast cell count and extravascular eosinophils if they were present. The other features (plasma cells, giant cells and areas of very high mast cell numbers) showed an irregular distribution both between preparations and on the same slide but if say four different preparations were studied, then all these would be found even if a single preparation did not show all the features together.

The mast cell troughs are real since successive days give very low values. The similarity of the mast cell peaks in position, time and relation to blood counts, between calf 152 and Charollais I gives further confirmation. Samples taken at the same time from different flanks gave identical elevated values (Day 79 calf 152) and it should be remembered that the samples were taken from a radius of about 5".

Mitoses of mature mast cells were seen on only one occasion in the preparations (on Day 85 Calf 152) and it seems unlikely that multiplication of the existing populatio was responsible for the rapid increases. Asboe-Hansen and Levi (1962) found that only about 1% of the nucei of mast cells in connective tissue below carcinogen-induced epidermi papillomas were labelled with tritiated thymidine within a period of 0·5-20 hours. The dense granules often obscure the nucleus and Allen (1963) has emphasized that to demonstr mitotic figures in mast cells special staining methods which show the nucleus in greater detail should be used. In this context Bloom (1963) while affirming the extreme rarity of mitoses normally seen in mature mast cells pointed out that few mitoses were seen in the normal liver and yet when stimulated these cells were capable of division at a high rate.

The increases are more likely to be due to the differentiation of existing precursors and in fact cells at all stages of maturation have been found. The age of a cell can be deduced from the morphology and especially the state of granule synthesis, from young cells exhibiting few granul to mature cells with dense granules and pronounced metachromasia as the degree of polysaccharide sulphation increases (Radden 1962).

The most widely accepted idea is that mast cells develop from the so-called perivascular mesenchymal cell a term almost synonymous with lymphocyte, and this work would tend to support such a concept. A maturation time of 100 hours has been suggested (Miller and Whitting 1964), however, the results presented in this thesis would suggest that the cells are capable of differentiation and maturation in shorter periods than this.

The reason for the stimulation must depend on the function of the mast cell within the body, such as a mediato

of tissue repair after damage or as a non-specific defence mechanism to toxic substances in the tissues; probably both are important and part of the normal inflammatory picture. It can be seen from the evidence presented that the increases take place at times of crisis in the animal, either when the blood counts of the animal are decreasing rapidly, or during the periods when the counts are low and the animal is attempting to recover. The calves undergoing the prolonged period of low dosage (Charollais I and 152) before being given bracken at a lethal level, exhibited a small increase 10 days after the dose increase. The animals whose peripheral blood counts recovered, frequently did so after a mast cell increase associated with a circulatory heparin release. This does not suggest a harmful role and no haemorrhages were visible at these periods.

It was seen that not all the mast cell increases were followed by the appearance of circulatory heparin and histamine, but it is true to say that all the heparin and histamine releases observed were preceded by mast cell incre
It is possible that this circulatory appearance is an oversp
phenomenon due to a particularly massive or sudden mast cell breakdown, or to the inability of the surrounding cells to respond and mop up the mast cell products locally before they reached the circulation. The heparin releases, as many authors have suggested, probably have a predominantly tissue function and a circulatory appearance represents an unusual phenomenon. Neither does it seem that heparin is the "natur
anticoagulant", however, it is possible that the localized anticoagulant activity may be of importance in preventing the thrombo-embolic disorders and consequent vascular damage which are a feature of some immediate hypersensitivity react
and the Schwartzman phenomenon.

The release of heparin in the tissues possibly has a dual purpose, firstly, the stimulation of the local phagocytic fibroblast population and the provision of material which can be used to synthesise new connective tissue; and secondly the clearing of toxic material by a non-specific action and the stimulation of phagocytosis and subsequent clearing of such material.

Jansen et al.(1962) have shown that intravenous commercial heparin produces a lymphocytosis in the calf, chiefly by an increased release of lymphocytes via the thoracic duct. The mechanism of action is not clear, although too rapid to be accounted for by new production. No analogous increases have been observed in the bracken poisoned calves. These authors point out that heparin has been shown to prevent the A.C.T.H. and cortisone induced eosinopenia and lymphopenia in man, the dog and the rat.

The appearance of heparin or rather heparin type anticoagulant, the effect of which could be neutralized with protamine sulphate, was clearly shown in the whole blood The plasma test did not consistently reveal parallel increase although an increase in the authentic heparin added was reflected in the test readings. Possibly the natural mast cell heparin released is not present as free heparin in the plasma but attached to the cells. Heparin has been shown to cause the clumping of leucocytes if used in too high a concentration and also to inhibit the leuko-agglutination reaction (Walford 1960). It is normally used in much lower concentrations in the isolation of leucocytes than is found in the blood of bracken poisoned animals. The heparin may also be attached in some way to the surface of red cells.

The heparinoid mucopolysaccharides have a high affinity for proteins, especially basic proteins, and the

antithrombin effect is exerted through an albumin-heparin complex. Heparin in the tissues exists in tight combination with protein and extraction is not easily achieved; the lipid clearing factor action is also mediated through a heparin-protein complex. Heparin also inhibits the enzyme ribonuclease and possibly through this affects cell division (Roth 1953).

The release of circulatory histamine has been shown to occur at the same time as some of the mast cell increases, and perhaps as suggested by Riley (1962) has the primary effect of preparing many more connective tissue cells than are normally available in the reticulo-endothelial system to receive heparin. Possibly release of histamine, whether or not a circulatory increase was detected, was important as a mediator of the phagocytosis seen in the animals at the times of the mast cell increases. Effects such as salivation and staring coats, which might be produced by histamin release, have been noted in bracken poisoned calves and it is likely that the large numbers of eosinophils observed in the tissues particularly at the times of mast cell increases are due to the release of histamine, although so far in this work there has been no observation of eosinophils showing any marked attraction to the mast cells themselves as they do to giant cells.

The argument as to whether these heparin and histamine releases are beneficial or harmful would seem to depend upon the degree of reaction and the capacity of the tissue cells to respond. A localised release is probably a necessary part of defence and repair, but constantly repeated inflammatory activity could result in irreparable tissue damage. A systemic overspill in the case of heparin may increase the risk of haemorrhage although unlikely in itself to be a primary cause, while histamine may be partly

responsible for the intestinal malfunctions (intussusception, tenesmus) seen in animals described in this thesis. Intestinal absorption of endotoxins discussed in Section II might have an important effect both on that site and systemically, since Urbaschek and Versteyl (1965) found that endotoxin had the effect of greatly raising the sensitivity of smooth muscle to histamine. These authors suggest that this phenomenon may play an important part in phenomena such as the Schwartzman reaction. The laryngitic type of death reported in young calves (Evans W.C.1961) may be due to a massive release of histamine, producing similar effects to guinea-pig anaphylaxis. As well as the effect on smooth muscle the histamine must have a generalized effect on vascular permeability.

The phagocytosis of the shed mast cell granules has been observed at the times of increase but the mechanism of disruption is not clear. The mast cell involvement in hypersensitivity reactions appears to be mediated by antigen-antibody reaction on the surface of the mast cells. Does a similar mechanism occur during the bracken poisoning syndrome?. The involvement of the mast cell in inflammatory processes is perhaps always mediated by an immunological mechanism if the concept of Boyden (1964) is correct. General cell damage may cause the release of cell components not normally circulating but to which antibodies already exist, although possibly at very low levels. The resultant combination of the two may set into motion the well known sequences of the inflammatory reaction.

The stimulatory influence causing the increase of the mast cell population may be antigenic in nature. Burnet (1964) has concluded from the evidence available that the small lymphocyte, the plasma cell and the mast cell can

be derived from a common precursor, i.e. the large lymphocyt (or blast) commonly found in the lymphoid tissue. Also the two functional cells may almost certainly be derived from the small lymphocyte. If as he suggests all these cells carry the same genetic potentialities of immune reaction, and plasma cells are stimulated by the presence of antigen it is surely possible that mast cells may also be stimulated Ginsberg and Sachs (1963) found that the differentiation of thymus cells to mast cells was enhanced in cultures infected with Malony virus. (During very preliminary attempts to culture in vitro the small samples of subcutaneous connectiv tissue from the calves, one specimen infected by a fungal growth contained great numbers of mast cells).

This possibly fits in with the concept of the mast cell as the non-specific mediator of resistance and the plasma cell as the specific, suggested by Higginbotham (1963 and may be a basically primitive form of resistance, since mast cells are found in the very simple forms of life. In this way the mast cell increases of the bracken poisoned animal may represent a last line of resistance. The cells may be stimulated by tissue breakdown, if so, is this becaus the breakdown products are antigenic or merely because they provide material for the synthesis of the characteristic granules?

The function of the mast cell as a part of the normal mucopolysaccharide cycle is possibly exaggerated, especially as phagocytosis of granules is not normally seen, after all bearing in mind Higginbotham's concept, one can hardly draw a parallel that a function of plasma cells is to provide new protein to phagocytic cells. Ringertz (1963) has expressed doubts as to whether heparin would serve as a precursor for the synthesis of ground substance polysacchari

The mast cell increases were always accompanied by at least one or more of the other manifestations of tissue activity of a type characteristic of immunological reactions. The degree of these changes must indicate their importance in the pathogenesis of the condition. These phenomena, lymphocyte and mononuclear perivascular concentrations, perivascular plasma cells and Giant cells were particularly prominent in the animals undergoing the long period of low dosage before receiving the lethal amount.

The lymphocyte and mononuclear concentrations were frequently seen in the bracken poisoned calves especial in the days of mast cell increases. Although these cells ar standard inflammatory cells, no pre-invasion of neutrophils was seen in any of the calves. This is a particular feature of delayed type hypersensitivity reactions (Humphrey and White 1964) and it is perhaps significant that many of these cells were in the process of changing to plasma cells. Sinc a generalized bacteraemia was not likely to have been presen in the calves, it may be asked what was the antigenic stimulation causing mass differentiation?

The giant cells observed in calves 152 and Charolla I are particularly noteworthy. Some multinucleate cells are normally found in spleen, lymph nodes and thymus and are produced by tissue culture of these organs or blood leucocyt (Weiss and Fawcett 1953).

Multinucleate giant cells have been observed by Shionoya et al. (1965) after the destruction of elastic fibre by the enzyme elastase and these workers have suggested a phagocytic function. Although the cells in the bracken poisoned calves appear to be much bigger and contain many more cell nuclei than the cells photographed by these workers

Particulate matter has been observed in the centre of the cells seen in the calves, but did not have the appearance of phagocytized material. Shionoya et al. suggest the need for mesenchymal stimulation brought about by some other factors. The nature of this stimulation may be immunological since Stein et al. 1965 have shown that auto-antibodies against partly degraded elastin exist in the normal human sera. They suggest that as elastin is an insoluble structural protein, its soluble derivatives might not be recognized as self by the immunologically competent cells.

The immunological function of giant cells possibly as producers of antibody would seem to be a more logical explanation. The cytoplasm of the cells is rich in R.N.A., since the cells stain intensely with the pyronin stain, occasionally the cells were seen surrounded by eosinophils in the manner of sensitized macrophages described by Spiers (1964). The part of eosinophils in the inflammatory response is well known and it is likely that these cells are capable of protecting the animal from the effects of histamine etc. The plate in this thesis shows the congregation around a giant cell, this is possibly a manifestation of chemotoxis to histamine released by an allergic reaction. The cells may have trephocytic function since in the animals observed they do not seem to be attracted to mast cells, perhaps in a manner analogous to the sensitized macrophages of Spiers they are responsible for carrying the processed antigen-R.N.A. complex. Tuberculosis is known to be a condition in which delayed hypersensitivity plays an important part and giant cells are found as a prominent feature of chronic tuberculous lesions. (Lewis 1927).

In view of these findings it was perhaps worthwhile to consider the possibility of immunological reactions in the pathogenesis of bracken poisoning. It was thought that

the following features were difficult to explain on the basis of a cumulative mitotic poison.

A 20 day period is consistently observed in the laboratory before the blood counts begin to fall, irrespecti of the final outcome or severity of the leucopenia. Does this mean that the effect depends on some reaction of the animal rather than directly on the amount of factor in the bracken fern?

Increasing the dose during a period of blood count fall does not seem to accelerate the effect, since the count have been observed to recover before falling again. This could be some kind of immunological paralysis. France (1964 reports that a heifer, bedded on bracken as a calf, showed clinical symptoms and thrombocytopenia after eating bracken for only 3 days; this might be due to presensitization.

An animal has been reported (Carpenter et al. 1950) which appeared to become resistant to large quantities of bracken given for prolonged periods. Similar quantities of the same bracken were sufficient to kill comparable animals, this possibly indicates some kind of resistance.

In the field bracken poisoning is frequently seen to appear in stock recently introduced to a bracken infested farm, stock reared on the farm do not appear to be so suscep tible. (This is no more than a general impression).

These phenomena are possibly explicable on the basis of immunological mechanisms, and the cellular reaction would suggest that the dominant manifestation is delayed hypersensitivity. The relationship of delayed hypersensit-ivity to immediate or antibody mediated hypersensitivity is not fully understood, although it has been suggested that delayed hypersensitivity precedes the appearance and is a necessary stage in the development of antibody production.

Possibly the sensitized cell has acquired the capacity to react but not to secrete antibody. It has been shown that the different manifestations of allergy may be developed to different components e.g. the immediate reaction was elicited by the capsular polysaccharide of Streptococcus Pneumoniaes, while a delayed type reaction was given to nucleoprotein. Delayed hypersensitivity rather than circulating antibodies accounts for the tissue lesions of most infectious diseases and is almost certainly the main agent responsible for the homograft rejection. (Humphrey and White 1964).

Drug sensitivities have been described by many workers and ascribed to immunological reactions, whether or not circulating antibodies are detectable. Two types of reaction have been seen (Heck 1955); an acute reaction with agranulocytosis, chills, fever and mucosal ulceration, and a more chronic type of leucopenia. He interprets the first as an allergic or sensitivity reaction and the second probably a result of toxic effects on bone marrow cells. The mechanisms of drug sensitivity in some cases do not appear to be simply a result of direct drug combination with the leucocyte, and a subsequent immunological rejection of the cell (Walford 1960). In some cases, circulating factors have been primarily implicated, although cell rejection may be the end result. A scheme has been suggested for a mechanism leading to hypoplasia and finally exhaustive atrophy of the bone marrow (Walford 1960 taken from Moeschlin 1955).

It is possible that similar mechanisms are involved in the pathogenesis of bracken poisoning, if the active principle enters the body and combines in some way with a cell or a cell constituent. (In short, a Hapten type of mechanism). This would explain the tissue reactions seen

and the length of time taken for these to develop, since the sensitivity would take time to build up. It would also seem to explain the particularly pronounced tissue reactions seen in the calves receiving the "presensitizing" dose of bracken. The susceptibility of calves compared with other species may be due to the particular immunological reactivit of the bovine, since this species like man is known to be particularly prone to allergies. Nevertheless, in such a complex syndrome it is quite possible that both immunologica events and toxic reactions play important roles in the aetiology of the condition.

Although thrombocytopenia and circulatory anti-coagulant may contribute to the widespread haemorrhages seen in the terminal phases, several authors are of the opinion that even with thrombocytopenia, haemorrhage does not occur unless there is capillary damage (Bedson 1922, Macfarlane 1941, Ackroyd 1949 a and b ). Thrombocytopenia and petechial haemorrhages have been caused by Sedormid, by a mechanism in which the drug in combination with the plate-lets of susceptible subjects, induces a state of sensitivity to further amounts of the drug. Ackroyd (1949 b ) suggested that some other factor, possibly allergic in nature and connected with endothelial cells, is necessary to precipitat haemorrhage, even in the presence of thrombocytopenia induce by the drug.

This type of reaction while an interesting possibil does not explain the similarity of bracken poisoning to irradiation effects and it would be of great interest to see if immunological mechanisms operate after irradiation. There are indications that they do and are of great importan

The results of Pugh et al. (1963) are of direct relevance and in summary are presented as follows:-

Marrow cells taken at intervals from rats irradiated by intravenous injection of $P^{32}$ were injected with adjuvant into rats of the same strain; when subsequently challenged with 1mc./Kg. $P^{32}$ intravenously, the recipients of the irradiated marrow cells developed a significantly increased granulocytopenia compared with control rats injected with normal cells.

The suppression of the immunological response in 1·5 mc.$P^{32}$ irradiated rats with Chlorambucil (10mg./Kg. day and 5 mg./Kg. day 2) was shown to lead to granulocytopenia and thrombocytopenia of less severity and duration than in controls not given Chlorambucil.

Spleen cells from irradiated and control donors were given intravenously to new born rats. When at 12 weeks of age these rats were subsequently challenged with 1mc./Kg. $P^{32}$, the degree and duration of thrombocytopenia was markedly accentuated in the animals which had received the irradiated cells. It was suggested that neonatal tolerance to irradiation-induced antigens in the spleen afforded protection to the lymphoid tissue, and upon irradiation the immunological potential was unimpaired.

It was suggested that the ionizing radiations induce new antigens in cells, these are recognized as such and the consequent immunological rejection results in superimposed immune injury to the cells in which the new antigens reside. These workers also report that alkylating agents may similarly cause the formation of new antigens in cells.

Russian workers (Klemparskaya et al. 1961) have made an intensive study of the auto-sensitization resulting from irradiation; they point out that similarities exist between allergic states and irradiation effects. These are decreased body temperature and fever, decreased complement

titre, blood coagulation disorders, widespread petechial haemorrhages, disturbances of gastro-intestinal function and the presence of a latent period etc.

They were able to show the following facts.

(1) Irradiated animals react severely to bacterial infection but not so much to the bacteria as to the tissue disintegration products produced by the bacteria. This takes at least 3 days to develop and auto-antibodies appear at this time.

(2) In animals given repeated irradiation, the titre of antibodies increased with the resistance of the animal to the irradiation.

(3) The appearance of leucocytolysines precedes the development of leucopenia after irradiation, these also appear after the injection of homologous intestine.

(4) These authors stress the importance of intestinal damage, the toxicity of homologous intestine homogenates, especially the mitochondrial fraction was shown and the effects produced were similar to irradiation. Homosensitization with this fraction made the animals very sensitive to irradiation.

These mechanisms may be sufficient to explain many of the tissue manifestations, but they may also be contributory to the fundamental bone marrow damage, and an explanation of the similarity between irradiation and radio-mimetic chemicals may be possible on these grounds. Both irradiation or chemically induced circulatory cell decreases appear to proceed in two phases, the initial phase being the immediate transient decrease in lymphocytes and platelets and the second phase being the granulocytopenia and possibly thrombocytopenia. (Bacq and Alexander). Bracken poisoning as it normally occurs in the bovine, appeared to produce only the second effect, but an induction of the first effect will be

discussed in Chapter II. Some of the radio-mimetic chemical at low doses exert either one or the other effect. Is it possible that the second delayed effect is at least partly an immunological one? It is interesting to speculate if Pugh et al.( in the work discussed previously) had used Myleran, which seems to have predominantly the second effect instead of Chlorambucil, would the irradiation effect have been suppressed or enhanced.

Two possible mechanisms can be envisaged. Firstly a Haptenic combination with either the cell surface or some cell constituent, or in the case of irradiation a direct physical effect on the macromolecules of the cell; both mechanisms causing the particular substance to become sufficiently changed to be recognized as antigenic. This is possible, and need not necessarily be mediated by antibodies since much cell damage is probably mediated by delayed hypersensitivity reactions. This would perhaps explain the latent period of development, although in fact delayed hypersensitivity appears to be quicker to develop than antibody production. Possibly this Hapten type of mechanism acts against all cells (or the haemopoietic stem cell described by Lewis and Trobaugh 1964); but the bone marrow and intestine in particular show effects because a high rate of replacement is needed. In this context, antibodies against burnt skin have been demonstrated (Kantor et al. 1965).

A second possible mechanism may involve the leakage or exposure of a cell component not normally available. Klemparskaya et al. (1961) have described the particular toxicity of the mitochondrial fraction and in fact mitochondrial damage has been observed after irradiation (Bacq and Alexander 1961). Possibly antibodies directed against subcellular components are developed, and the particular

importance of the intestine is that the effect is enhanced by the local leakage of bacterial endotoxins. Bitensky (1963) in discussing the cytotoxic effects of antibodies has suggested a possible mechanism for this development of auto-sensitivity. She visualized that structural lipids of the cell are normally bound tightly to protein and as such are recognized by the reticulo-endothelial system, but an unmasking of the lipid moiety was an early response to abnormal conditions. She suggested that this is a possib mechanism for the potentiation and prolongation of auto-immune diseases, and that lysosomal damage may be of particular importance. The development of antibodies against slightly damaged nuclear protein has been demonstrated as a possible mechanism in the development of lupus erythematosus (Humphrey and White 1964).

It might be argued that irradiation has been shown to suppress the immunological response, but according to the Russian workers, this is not so much an inhibition as a saturation with auto-antigen. Dixon and McConohey (1963) showed that the response to antigen given slightly before irradiation is actually enhanced. The presence of some degree of sensitization against subcellular components, not normally present at a level high enough to suppress the "forbidden clones" of Burnet, may be visualized; and perhap the occasional inevitable tissue injury is sufficient to maintain some degree of auto-sensitization similar to that suggested by Boyden. A mass breakdown or mass availability of auto-antigen to which the body is already presensitized (although possibly at a very low level) would cause in effec a secondary response resulting in widespread cell damage.

Anti-R.N.A. auto-antibodies have been produced experimentally (Burnet 1963) and have been described as

existing in the normal animal (Barbu and Dandeu 1963 and Serra et al. 1965). It would be of great interest to find if these antibodies were concerned with the suppression of cell mitosis in the manner of chalones, since Serra et al. found that although these antibodies were present in the sera of normal healthy subjects they were absent in the sera of carriers of malignant tumours. Possibly relevant to this context is the observation by Maurice and Jeanrenaud 1963 of the production of a bone marrow mitotic depressive factor by local splenic irradiation.

In summary, it may be said that the work has indicated the importance of immunological mechanisms in the pathology of bracken poisoning; and by analogy, in irradiati and other radio-mimetic conditions. The possibility of cortisone therapy suppressing the tissue manifestations should be considered for future work. The relationship of the tissue reaction and the bone marrow damage has not been resolved but it would perhaps seem that immunological mechanisms are of fundamental importance in both respects.

# CHAPTER II.

## STUDIES ON HYPERSENSITIVITY TO BRACKEN.

## Introduction.

The studies on the tissue reactions observed during the course of bracken poisoning, discussed in the previous chapter, seemed to provide strong evidence for the involvement of immunological mechanisms in the aetiology of the condition. Furthermore, the presence of giant or multinucleate cells in the tissue samples of calves which had been on bracken for a prolonged period of 10-12 weeks, was very reminiscent of tuberculosis; a condition in which delayed hypersensitivity reactions play an important part, and where these cells form a striking feature of the lesions. (Lewis 1927). If the deductions were valid, it seemed possible that animals having had small amounts of bracken might be sensitized to further contact. It was decided to test this, using a Mantoux type technique, as for T.B. infections, that is the intradermal injection of small quantities of potentially antigenic material, and to observe the subsequent reaction. A positive reaction would be indicated by a measurable swelling at the site of injection after 24-48 hours.

For injection an ethanolic extract of the dried frond was prepared, since Evans *et al.*(1958) showed this to be active in producing the bovine haemopoietic syndrome.

## Preparation of an ethanol concentrate.

Eight hundred grams of the June 1964 dried bracken were placed in a five litre round bottomed flask fitted with a reflux condenser. This was heated with $3\frac{1}{4}$ litres of ethanol on an electric isomantle with occasional shaking until the ethanol had just started to boil,

the supernatant was then filtered through muslin and the filtrate stored for at least 24 hours at 3°C. The extraction was repeated four times increasing the boiling sequentially to ten minutes, half an hour and finally two periods of 1½ hours each; assuming that more of the active principle came out in the first batches this would avoid excessive heating. Evans et al. (1958) found about half the activity with respect to the bovine was extracted by bringing the ethanol to the boil three times.

The extracts after standing in a cold room, were filtered through Whatman No.540 filter paper to remove the cold insoluble phospholipids, which precipitate down. This filtrate was then concentrated to 240 mls. (300mls. /Kg. dried bracken) under reduced pressure in a rotary film evaporator. The resultant thick green solution was used for all subsequent steps.

Initial Skin Test and Method of Measurement.

Three calves had been used to test the activity of the anode, cathode and neutral fractions after electrodialysis of an aqueous extract of the fresh bracken frond (Humphreys 1964 unpublished). None of the calves showed significant changes in blood counts. The skin test was performed 12 days after the last dose was given, and 0•2 mls. of ethanol concentrate was injected intradermally in the neck of each calf.

Immediate swellings were observed at both these and control ethanol injection sites, these swellings subsided after about three hours; but after 48 hours the bracken extract injection sites were marked by large swellings while the control ethanol sites were barely detectable. Samples of subcutaneous connective tissue taken

from underneath the bracken-induced swelling of one animal revealed concentrations of lymphocytes, large mononuclears and plasma cells.

In view of these results it was felt that the line was promising enough to warrant further investigation, but obviously calves which had had no previous contact with bracken would have to be tested in order to eliminate the possibility of a non-specific reaction. From the experience gained in this initial test, and in order to obtain a more quantitative basis for results, the following procedure was adopted for all subsequent tests:-

The test material in 0•2 or 0•3 ml. of ethanol was injected intradermally using an ordinary 1 ml. syringe by holding a fold of skin in the left hand and inserting the needle parallel to the animal's surface in the same direction as the fold.

The subsequent reaction was measured at the appropriate time using a pair of bovine tuberculin test calipers. The reaction was measured by placing the calipers behind the swelling and then withdrawing them so that the swelling passed between the jaws; the fold of skin containing the swelling being held in the left hand. This was performed several times on every site and the highest consistent value noted. The thickness of the normal skin at the site immediately adjacent to the swelling was deducted to give the actual reaction size (mm.). Although measurements were taken, the technique suffered from difficulties such as site variation, and was really more qualitative than quantitative. The tests were performed using ethanol, since all the fractions were soluble in ethanol, thus avoiding the difficulty of saline suspensions and possible loss of activity on the glassware.

However, this had the disadvantage that no immediate hypersensitivity reaction could be seen, since the reaction to ethanol would prevent its detection.

Second Skin Test.

Workers in this department have often obtained variable results in the fractionation of the plant, when using precipitation techniques as part of the separation methods, so it was decided as a first step to separate the ethanol concentrate into polar and non-polar fractions, but avoiding the formation of a precipitate. In an initial attempt at a benzene-water partition, an interlayer-precipitate was formed and both soluble fractions were found to be inactive. Further use of Benzene was abandoned and the following method was evolved.

Distilled water to a final concentration of 20% by volume was added to the ethanol concentrate, thus raising the polarity. The solution was then extracted four times with twice the volume of n-Heptane; by the final extraction the overlying layer of n-Heptane was clear. Ethanol and n-Heptane are normally miscible but the addition of the water forced the separation of the two phases. The n-Heptane soluble fraction was evaporated to dryness under reduced pressure and taken up in a volume of ethanol equivalent to the original ethanol concentrate.

0•2 mls. of this were injected into the anode, cathode and neutral calves used initially; and as a control experiment into animals of a similar age from the College Farm. As far as could be ascertained, these animals had no previous contact with bracken, having just been draughted in from a lowland area and still confined in a covered yard.

The results are represented by the following table.

| Calf. | Skin readings (mm.). | | 48 hour Reaction (mm.). |
|---|---|---|---|
| | pre-injection. | post-injection. | |
| Anode. | 6 | 16 | +10* |
| Cathode. | 6 | 26 | +20* |
| Neutral. | 5 | 15 | +10* |
| X1818. | 7 | 11 | +4 |
| C96. | 6 | 10 | +4 |
| C94. | 4 | 8 | +4 |
| 459F. | 7 | 12 | +5 |
| C93. | 5 | 10 | +5 |

* Reaction.

Forty-eight hour reactions of more than +6 were assumed to be positive (*) and reactions of less than this figure negative. Occasionally the needle insertion caused localised bleeding with subsequent clot formation, which increased the measured skin thickness to +5 or 6. For this reason values of +6 were designated as "possible"(?).

It was felt that these results were highly encouraging since the calves sensitized by ingestion of bracken extracts reacted unmistakably, while the calves having no previous contact with bracken only produced reactions comparable to pure ethanol. The extract used for the skin t must therefore contain some factor or factors which produce an allergic reaction, and it would seem possible that the test could be used as a bio-assay in an attempt at the

isolation of the active principle.

It was decided to follow this line of work with the following objects in view:-

(1) To investigate further the role of hypersensitive phenomena in bracken poisoning; (2) to determine the relationship of the bracken haemopoietic factor and the allergic factor; which might lead in turn to the discovery of the unknown haemopoietic factor itself.

The following section describes the extraction steps and the separation procedure, culminating in the isolation of a highly active fraction. The results of administration of this to a calf *per os* will be given and discussed together with additional information about the sensitivity state.

Extraction of the n-Heptane soluble fraction.

If the agent producing the allergic reaction and the cattle haemopoietic factor were the same, then it was surprising that the activity went into n-Heptane, since the bovine syndrome had been produced by hot water and dilute acetic-acid extracts (Evans W.C. 1959). However, in many cases these were not true solutions but suspensions containing particulate matter. The factor also seemed to have basic properties since it was extractable by ether from alkaline aqueous solutions but not from acid solutions (Evans W.C. Personal communication).

The ethanol-water phase during the n-Heptane extraction was at pH 5•5, so it was possible that a weakly basic compound would go into the n-Heptane; it was therefore decided to extract the n-Heptane solution with a mixture of ethanol (80%) and 0•1N hydrochloric acid (20%). The n-Heptane solution was extracted four times with half of its volume of this mixture, the lower ethanol-acid phase being clear by the last extraction.

The ethanol-acid extract was evaporated and taken up in a quantity of ethanol equivalent to the volume of the original ethanol concentrate. Using 0•3mls. of this for injection, reactions of +10, +10 and +7 mm. were produced in the anode, cathode and neutral calves respectively. It was not possible to run paper chromatograms with the ethanol-acid extract since there was still a considerable amount of pigment present, accordingly the extract was taken and evaporated under reduced pressure until only about 1ml. of the acid was left. This caused the pigment to precipitate and stick to the sides of the vessel. The aqueous material, rather oily in appearance, was removed and the precipitate washed with 5mls. of 0•1N

hydrochloric acid; this acid soluble fraction was shown to be capable of producing a skin reaction (Fig.16 ).

It was therefore thought worthwhile to investigate the compounds present in this solution since the absence of pigments readily permitted examination by paper chromatography.

Paper Chromatographic investigation of the acid soluble fraction.

Methods.

Solvent Systems.

Several solvent mixtures were tried including n-Butanol-water-·880 ammonia (172:18:10); n-Butanol-N sodium acetate-N hydrochloric acid (7:120:60) and n-Butanol-water (172:28), but it was found that the most satisfactory solvent was n-Butanol-glacial acetic acid-water in the proportions by volume of 120:30:50. This gave the best separation of the acid soluble mixture with virtually no streaking. A fresh batch of solvent was made up for each run.

Spray Reagents.

Bromo-cresol Green.

This is merely a pH indicator obtained by dissolving Bromo-cresol Green (0·05% w/v) in ethanol; basic compounds give green or blue reactions, acid areas show up yellow. The background colour may be altered by a brief exposure to ammonia vapour, strongly acidic spots

are more sharply defined, while basic compounds not previously visible may become visible as the blue background fades.

Ninhydrin.

The classical amino-acid reagent was prepared by dissolving 0•2% ninhydrin w/v in acetone, 2% pyridine was added immediately before use. All α amino-acids react in the cold, usually within three hours, giving in the main purple colours. Compounds having secondary or primary amino groups usually react if the paper is heated in an oven at 105°C for 2-3 minutes.

Aniline-Xylose.

This was prepared by dissolving xylose (1gram) in 3 mls. of water, adding aniline (1ml.) and making up to 100 mls. with methanol. If the paper is sprayed, allowed to dry, and heated for 5-10 minutes at 105°C, as little as 10μg of most acids appear as brown spots.

Dragendorff reagent.

The reagent was prepared by boiling 5 grams of potassium bismuth iodide in 100mls. of distilled water containing 0•5mls. of concentrated hydrochloric acid, the solution was then allowed to cool and filtered. Heterocyclic bases give colours ranging from purple to orange (Bartley 1954), however the reagent shows considerable variation in sensitivity to different bases.

Ultra-Violet light.

Many compounds can be detected without chemical

## CHROMATOGRAPHY OF THE ACID SOLUBLE FRACTION.

(Butanol–Acetic acid–water.)

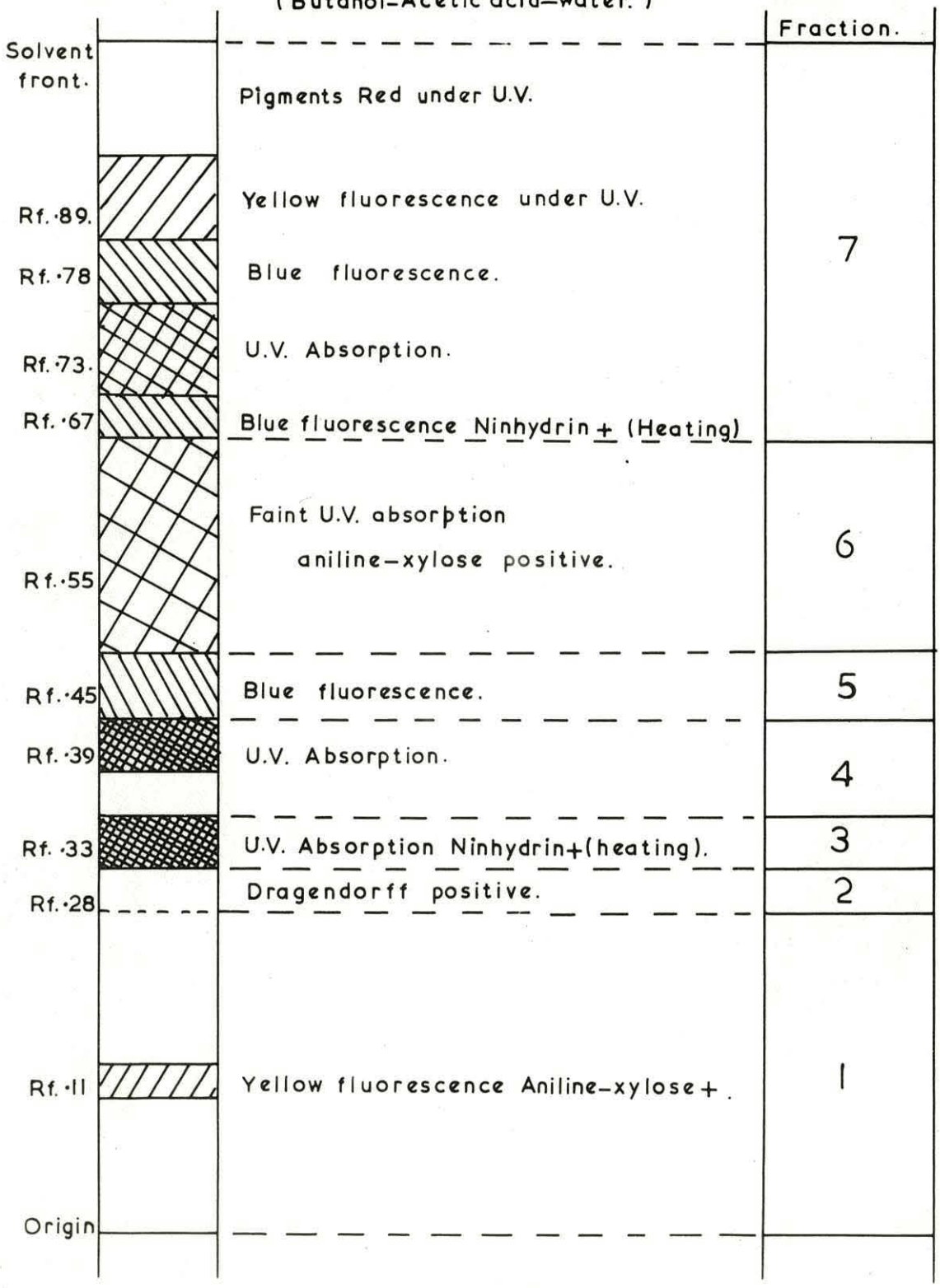



Fig. 14.

alteration by their fluorescence or absorption when observed under UV light; this procedure is invaluable as a preliminary step in the investigation of chromatograms; visible compounds are marked in pencil.

Summary of Chromatographic results.

Ascending chromatograms were run at room temperature in a tank measuring 16 x 7 x 20 inches using 400 mls. of the Butanol-glacial acetic-water solvent per run. The solvent front travelled 9-10 inches in 12-14 hours under these conditions. The results are summarized in Fig.(14 ).

Test of paper chromatogram eluates.

Preliminary investigations of eluates showed that the activity ran in the region Rf 0-0•45, so the following experiment was designed to locate this exactly.

20 mls. of ethanol concentrate were put through the procedure and the final acid soluble material evaporated down under reduced pressure to about 0•5 ml. and then made up to 5 mls. with ethanol. Four mls. of this were applied to a 10" wide sheet of Whatman No.3MM chromatography paper as a thin streak and allowed to run overnight for 14 hours. The chromatogram was then dried with a hair dryer, examined under U.V.light and the fluorescent and absorbant bands marked in pencil.

Four three-quarter inch vertical strips were then cut off the chromatogram and sprayed with Bromocresol Green, Dragendorff, Ninhydrin and Aniline-Xylose

reagents. The remaining chromatogram was cut horizontally into the bands marked in Fig.( 14 )., these sections were cut into smaller pieces and eluted with 3x 150 mls. of hot ethanol. These extracts were filtered, evaporated to dryness under reduced pressure and taken up in 5 mls. of ethanol These fractions, the original ethanol concentrate and the acid soluble material, were tested by the intradermal reaction using a calf which had had twenty-eight daily doses of an acetic acid extract of bracken frond, the last dose being 15 days before injection. (This calf which received the extract from 20 lbs. wet weight of frond / day did not show any marked effect in the blood counts).

The results are shown in the following table.

| Fraction. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Total acid sol. | EtOH conc. |
|---|---|---|---|---|---|---|---|---|---|
| Reaction 24 hour. | +4 | +5 | +2 | +1 | +3 | +2 | +2 | +5 | +5 |
| Reaction 72 hour. | +4? | +8* | +2 | +2 | +3 | +2 | +2 | +5* | +7* |

* Reaction. ? Possible reaction.

The reaction seemed to be exactly coincident with the Dragendorff positive compound in fraction 2. Since the cattle factor seemed to have basic properties, and bases such as the Vinca Rosea Linn. alkaloids are known to be radio-mimetic it was thought worthwhile to purify this Dragendorff positive spot and to test the pure compound for allergic reactivity.

Preparation of a large amount of the Dragendorff positive compound.

INFRA RED SPECTRUM OF DRAGENDORFF POSITIVE COMPOUND.

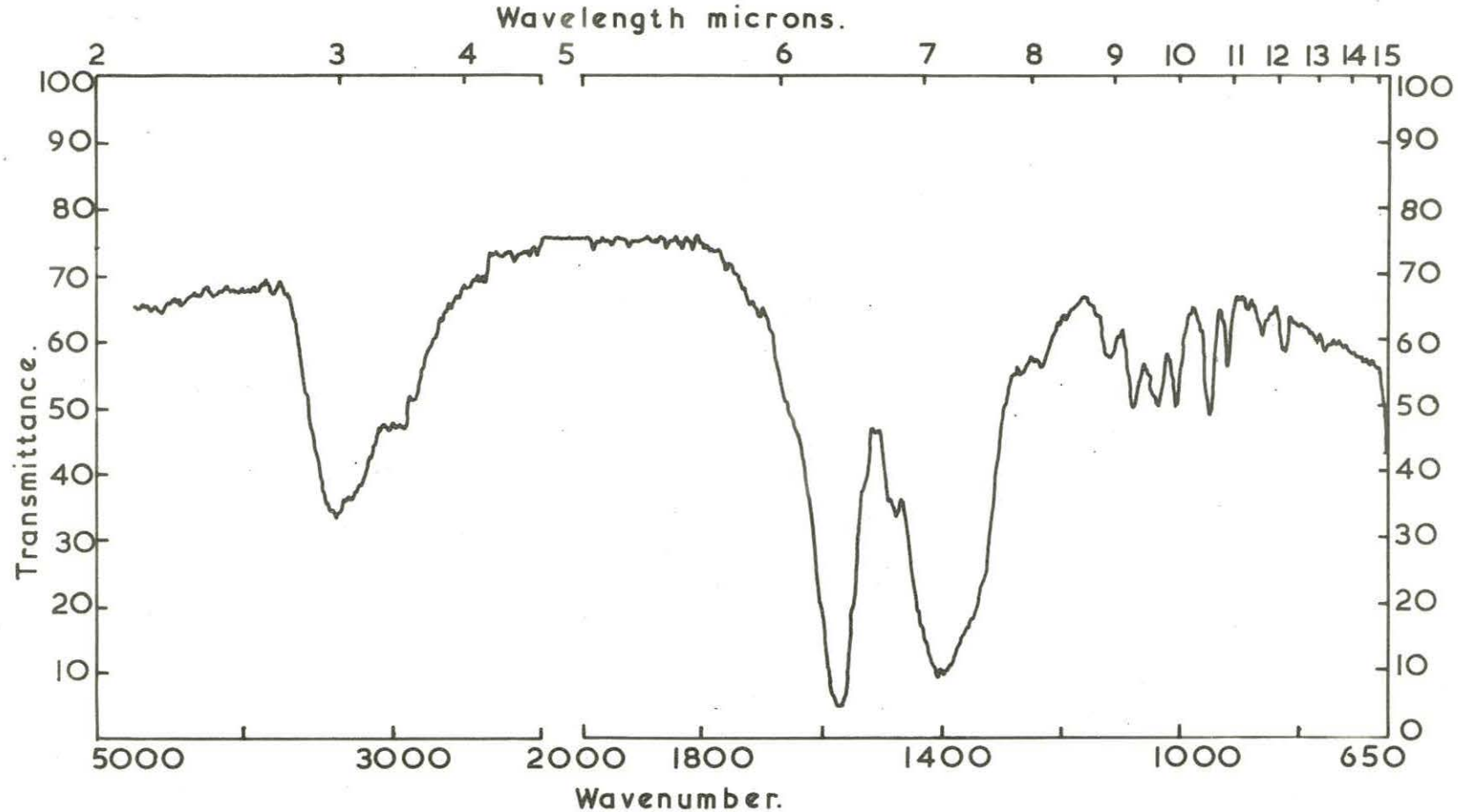



Fig 15.

One kilogram of June 1964 bracken powder was extracted with ethanol in the usual manner; the ethanol concentrate was processed through to the ethanol acid stage and this evaporated by hand to 10 mls. of aqueous residue. This was applied as thin streaks on ten 10" wide sheets of Whatman No.3 MM chromatography paper, and run 12-14 hours in the Butanol-acetic acid-water solvent. The chromatograms were dried, examined under U.V.light and a narrow vertical strip of each sprayed with Dragendorff reagent.

The bands corresponding to the Dragendorff positive compound were cut out, combined and eluted with hot ethanol. The eluate was concentrated and streaked on six more 10" wide chromatograms of 3MM paper; the Dragendorff positive compound this time ran with an Rf of 0•4 and the only contamination visible under U.V.light was an absorbing band running in front with an Rf of 0•5. The Dragendorff positive bands were cut out and eluted with hot ethanol, the extract was filtered and concentrated to 0•5 mls. when crystals were obtained. These were removed from the mother liquor by centrifugation and recrystallization from ethanol. A yield of no more than a few milligrams of slightly brownish material, becoming oily on gentle warming, was obtained. An infra-red spectrum of this compound was obtained using a potassium chloride disc, this is shown in Fig.(15 ).

When tested in the same calf as used for the original band, this pure compound failed to show any activity. The extraction and purification were repeated and again the pure compound was shown to be inactive.

It was concluded from these results that the activity was not due to the Dragendorff positive compound but rather than spend time in locating the other substances

## EXTRACTION PROCEDURE.

Dried Bracken 1964 (65 grams).

↓

20 mls. Ethanol Concentrate.

↓

5 mls. $H_2O$

Extracted with 4x50 mls n Heptane. → EtOH $H_2O$ Residue.

↓

200 mls. extract.

↓

Extracted with 4x100 mls. of EtOH (80 mls.) & HCl 0·1N (20 mls.) → n Heptane Residue.

↓

400 mls. extract.

↓

Concentrated to 1 ml. 5 mls 0·1N HCl. → Pigments Residue.

↓

0·1N HCl Soluble.

48 hour Reaction of:—

| | 0·2 ml. | | | 0·3 ml. | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H |
| | +8* | +6* | +7* | +7* | +12* | +15* | +10* | – |
| | +4 | +3 | 0 | +1 | +4 | +3 | +4 | – |
| | ?+6 | +5 | ?+6 | +8* | +12* | +16* | +11* | – |
| | +7* | +4 | +4 | +6* | +10* | +16* | +8* | +16* |
| | +10* | +5 | ?+6 | +4 | – | – | +8* | – |
| | +5 | +5 | +4 | +4 | +7* | +4 | – | +7* |
| | +8* | ?+6 | ?+6 | ?+6 | ?+6 | ?+6 | – | +7* |

* Reaction. ? Possible reaction.

Fig. 16.

present on the chromatograms, it was decided to leave the chromatographic examination at this stage and to re-examine the total n-Heptane fraction.

Investigation of the n-Heptane fraction.

Comparative studies showed that all the allergically reactive material did not go into the acid soluble fraction. Fig. (16 ) represents the results from all the animals tested with the different fractions of the procedure simultaneously; it can be seen that although no activity was ever found in the ethanol-water, after that stage the activity was divided. It was decided to find some fractionation method which would not split the activity and would be possible to use on a large scale, so that a calf could be given the allergic factor per os (purified as far as possible), in order to determine the relationship between the allergic factor and the haemopoietic factor.

The n-Heptane fraction was chosen as the starting material, since this contained all the detectable activity. It was decided to attempt fractionation using column chromatography with alumina as the adsorbant, this is commonly used for non-polar material and was cheap enough for use on a large scale.

Preparation of Alumina ($Al_2O_3$).

When purchased, commercial grades of alumina contain small amounts of alkaline material, this can be removed by washing with dilute acids, so the batches of alumina were treated in the following manner preparatory

to use.

Peter Spence type O "activated" alumina (2 kg.) was placed in a ten litre glass container, and 4450 mls. of distilled water added, this was followed by 450 mls. of concentrated hydrochloric acid ( S.G.1·18) to give a Normal solution. This was vigorously shaken periodically throughout one day and allowed to stand overnight. The supernatant was then removed at the pump, and six litres of distilled water added; after shaking vigorously the suspension was allowed to settle and the supernatant removed. This was repeated twelve times, over two to three days, allowing the suspension to settle at least half an hour before sucking away the supernatant at the pump. After the last washing, the alumina was transferred to a large porcelain basin and dried in an oven at 105°C for twenty-four hours. The powder was then divided into approximately 300 gram batches, put into "Sillex" evaporating basins and heated at 500°C for at least two hours, it was then allowed to cool and stored in polythene bags until ready for use.

Preparation of column.

The degree to which lipids and other non-polar materials are held by the alumina, is inversely related to the amount of water adsorbed on to the alumina, so the aqueous phase must be accurately controlled. The required amount of the fully activated (500°C heated) material was weighed accurately, placed in a porcelain basin and covered with n-Heptane. The volume of water needed was added dropwise to this from a pipette and distributed evenly by gentle crushing and mixing with a pestle. The final moisture content in all the column

runs was 6%.

The columns were made from glass tubing of a convenient bore fitted with a tap and normally about twice as long as the height of the column. A pad of cotton wool was placed in the bottom and n-Heptane added, the cotton wool was then pressed with a glass rod to remove bubbles. The alumina was added as the n-Heptane slurry, maintaining a large head of n-Heptane in the column, to ensure that the alumina falling through this settled as evenly as possible and packed uniformly. The level of the n-Heptane was not allowed to fall to the surface of the alumina until the mixture to be chromatogrammed was ready for application. Following normal practice, the material was applied in the first of the solvents to be used for elution, in as small a volume as possible. The mixtures were applied immediately and not allowed to stand (to avoid precipitation); if too concentrated a band of the n-Heptane solution was applied, the undissolved material tended to block the column and restrict the flow rates.

Run 1.

20 mls. of n-Heptane extract prepared from 2 mls. of ethanol concentrate were evaporated to dryness. The residue was taken up in 3 mls. of n-Heptane, 1·5 mls. of this was added by pipette without disturbing the surface, to a twelve gram column, 14 cms. long x 1 cm. diameter, containing 6% water. The mixture was allowed to run until level with the surface of the alumina, and the column then eluted at a flow rate of 1 ml./ minute with the following series of solvents. n-Heptane (50 mls.); 5% diethyl ether in n-Heptane (25 mls.); 10% diethyl ether in n-Heptan (140 mls.); diethyl ether (40 mls.) and ethanol (50 mls.).

The column was then extruded and extracted with hot ethanol. The substances in the mixture would have been eluted according to their degree of polarity, the more polar compounds coming in the later fractions; the gradual change in eluant concentration prevented any "mixing" effects and consequent column distortion.

The eluates were evaporated to dryness under reduced pressure and taken up in 1 ml. of ethanol. The fractions (0•3 ml.) were then tested on calf 154 (described in this thesis) on the 20/5/65 together with some of the original crude n-Heptane fractions.

The results are summarized in the following table.

| Fraction. | Pigments eluted. | 48 hr. reaction. | 72 hr. reaction. |
|---|---|---|---|
| 50 mls. n-Heptane | yellow band | +6? | +7* |
| 50 mls. 5% ether<br>25 mls. 10% ether | yellow+green band | +8* | +12* |
| 140 mls. 20% ether | pink+gray bands | +4 | +2 |
| 50 mls. ether | complex | +4 | +1 |
| 50 mls. EtOH | complex | +4 | +2 |
| Hot EtOH | Residue at top | +4 | +2 |
| Original sample | - | +12* | +12* |

* Reaction. ? Possible reaction.

Run 2.

Fourteen grams of alumina containing 6% moisture were used (column 16 x 1 cm.); 20 mls. of n-Heptane extract, equivalent to 2 mls. of ethanol concentrate, were evaporated to dryness under reduced pressure and applied to the column top in 2 mls. of n-Heptane. The results are summarized in the following table, the fractions in two mls. of ethanol being tested (0•3 ml.) (25/5/65) on the Aberdeen Angus calf described in this thesis.

| Fraction. | Pigments eluted. | 48 hr. reaction. | 72 hr. reaction. |
|---|---|---|---|
| 57 mls. of n-Heptane | Yellow | +7* | +7* |
| 50 mls. of 10% ether | Yellow+pink | +7* | +6? |
| 50 mls. of 20% ether | Complex | +4 | +3 |
| Ether + Hot EtOH | Complex | +4 | +4 |

* Reaction. ? Possible reaction.

From these results it appeared as if the activity was divided into two bands, in the first and second fractions or was concentrated in a single band at the end of the n-Heptane fraction and the beginning of the 5 or10% ether. It was decided to use a larger column and to fractionate the material which could be obtained with exhaustive n-Heptane elution.

Run 3.

A ninety gram column 30 cm. x 2 cm. diameter was set up. 20 mls. of ethanol concentrate were extracted with n-Heptane and the extract applied to the top of the column in 20 mls. of n-Heptane. The column was eluted with n-Heptan and the eluate collected as seven separate fractions; the column was then extruded and extracted with hot ethanol (250 mls.).

The results are summarized in the following table, all fractions being taken up in 5 mls. of ethanol and tested on the calf 154 (1/6/65), injections of 0·3 ml. being used.

| Fraction | Volume of n-Heptane. | | 72 hr. reaction. |
|---|---|---|---|
| 1 | 250 mls. | | +11* |
| 2 | 50 mls. | Pigment (yellow). | +3 |
| 3 | 50 mls. | | +4 |
| 4 | 50 mls. | | +3 |
| 5 | 50 mls. | | +3 |
| 6 | 50 mls. | Colourless oily material seen. | +12* |
| 7 | 250 mls. | | +4 |
| 8 | Hot EtOH extract of all residual material. | | +6? |

* Reaction. ? Possible reaction.

The reactive material appeared to be concentrated in two bands, both giving a strong reaction, one running before the yellow pigment band, the other just after .

Paper chromatography of Fractions 1 and 6.

Half of the reactive bands 1 and 6 were streaked on two 10" wide sheets of Whatman 3 MM paper and run in the Butanol-acetic acid -water solvent for 14 hours. After drying these were examined under U.V.light; nothing except a small amount of pigment running with the solvent front could be seen; narrow strips sprayed with Dragendorff reagent did not reveal any Dragendorff positive area.

The area between Rf 0•2 and 0•5 was cut out on each chromatogram and extracted with hot ethanol, since this contained the active band previously obtained from chromatography of soluble material of the acid-ethanol extract. The remaining parts of the chromatograms were also extracted. The extracts were evaporated under reduced pressure and combined, taken up in 2•5 mls. of ethanol and tested on calf 154 (30/6/65) 0•3 mls. being used.

The results are indicated by the following table.

| Fraction. | Chromatogram area. | 48 hr. reaction. |
|---|---|---|
| 1 | Rf 0•2-0•5 | +3 |
| 1 | Rest of chromatogram | +10* |
| 6 | Rf 0•2-0•5 | +8* |
| 6 | Rest of chromatogram | +3 |

* Reaction.

It is possible that the reaction from fraction Rf 0·2-0·5 represents the activity obtained on the chromatograms of the acid soluble material Rf 0·4; if the activities were due to the same substance, this would confirm that the activity was not due to the Dragendorff positive compound.

Run 4.

This was virtually a repeat of Run 3. using 100 grams of alumina and applying the mixture in 15 mls. of n-Heptane.

The fractions dissolved in 5 mls. of ethanol were tested on the Aberdeen Angus calf on the 4/6/65 and the effect of ·3 mls. of these is shown in the following table.

| Fraction. | Volume of n-Heptane. | 48 hr. reaction. |
|---|---|---|
| 1 | 200 mls. | +8* |
| 2 | 100 mls. } Pigment. | +6? |
| 3 | 100 mls. } Pigment. | +3 |
| 4 | 100 mls. | +8* |
| 5 | 230 mls. | +2 |
| 6 | Hot ethanol extract. | +6? |

* Reaction. ? Possible reaction.

Summary.

The n-Heptane extract could be fractionated using alumina columns, the main activity came with the earlier non-polar fractions, possibly a very slight amount of activity remained on the column in runs 3 and 4 with the larger columns. The activity was concentrated in two bands indicating the presence of at least two agents responsible for the hypersensitive reaction. The separation achieved was very great since the dry weight of the n-Heptane and 5% ether eluted material combined amounted to only 4% of the original ethanol concentrate and this contained pigments shown not to be active.

Preparation of large amounts of the allergic factors.

Since the separation procedure of the allergic factors was capable of being worked on a large scale, and the purification achieved so great, it seemed obligatory to test the relationship between the allergic factors and the cattle haemopoietic factor.

Five Kilos of June 1964 bracken fed over 5 days were known to produce a detectable change in the blood counts (see Aberdeen Angus and calf 154). It was decided to process about 10 Kilos of bracken since this was about the largest amount which could be processed in the time available, and yet would allow a reasonable surplus to counteract the loss by incomplete extraction and inactivation.

It was decided to test as one fraction everything that was eluted by n-Heptane and 5% ether in n-Heptane. Although this contained a good deal of pigment material not active by the allergy test, it was felt that such a step would ensure that the two highly active bands were completely eluted from the column; especially under the different conditions caused by the increase in scale.

Method.

A Quickfit glass column dimensions 1metre x10 cms. diameter, with its own supporting stand was used. The alumina rested on a perforated disc covered with a pad of cotton wool. To fill the column, five litres of n-Heptane were poured into the column, the required amount of alumina was added as a n-Heptane slurry while the liquid was vigorously agitated with a mechanical stirrer. The disturbance was sufficient to keep the whole amount of

alumina in suspension. The stirrer was then removed and the column allowed to settle.

Column Run 5.

9•6 Kilos of the June 1964 dried frond were extracted in the usual way and the extract concentrated to 1800 mls. 600 mls. of this were taken, 150 mls. of water added and the mixture extracted with 4x1500 mls. of n-Heptane. The n-Heptane extract was concentrated under reduced pressure in a rotary evaporator until no more n-Heptane came off. 450 mls. of n-Heptane were then added and the solution put on top of a 3450 gram column (6% moisture). The column was eluted with 12 litres of n-Heptane followed by 17•5 litres of n-Heptane containing 5% ether; a yellow band was completely eluted by the first solvent and a second yellow band by the 5% ether. The column was then extruded and the alumina extracted with hot ethanol.

The eluate and the extract were evaporated to dryness under reduced pressure in a rotary evaporator and then both fractions were taken up in ethanol.

Column Run 6.

The remaining 1200 mls. of ethanol concentrate were extracted initially in the same manner as Run 5, in two 600 ml. batches. The combined n-Heptane extracts were then reduced to dryness, taken up in 300 mls. of n-Heptane, and put on the top of a four Kilogram column. The column was eluted with ten litres of n-Heptane, followed by thirteen litres of n-Heptane containing 5% ether. The second yellow band was completely eluted. The column was extruded and extracted with hot ethanol.

This last column was overloaded in comparison to the previous columns, since it received twice the amount of n-Heptane without a corresponding size increase. The concentrated column eluates from Runs 5 and 6 were combined and taken up in 700 mls. of ethanol; while the combined residues from the columns soluble in hot ethanol were taken up in 1,100 mls. of ethanol, giving a thick green solution. These were tested on calf 154, using 0·7 of the eluate solution diluted to 1·1 mls. with ethanol and the column residue solution undiluted. 0·3 mls. of each were injected, along with 0·3 mls. of the original ethanol concentrate.

The following reactions were obtained.

| Fraction. | 48 hr. Reaction. |
|---|---|
| Column eluate. | +24* |
| Column Residue. | +10* |
| Original Ethanol concentrate. | +9* |

* Positive reaction.

It was concluded that the material eluted was very active (producing the largest swelling ever recorded by these tests), although the column had obviously retained some activity. This was possibly due to the column Run no.6. in which conditions were altered, however the increase in scale may have contributed, since there was possibly a residual activity in runs 3 and 4; with the smaller columns 1 and 2 no detectable activity was retained.

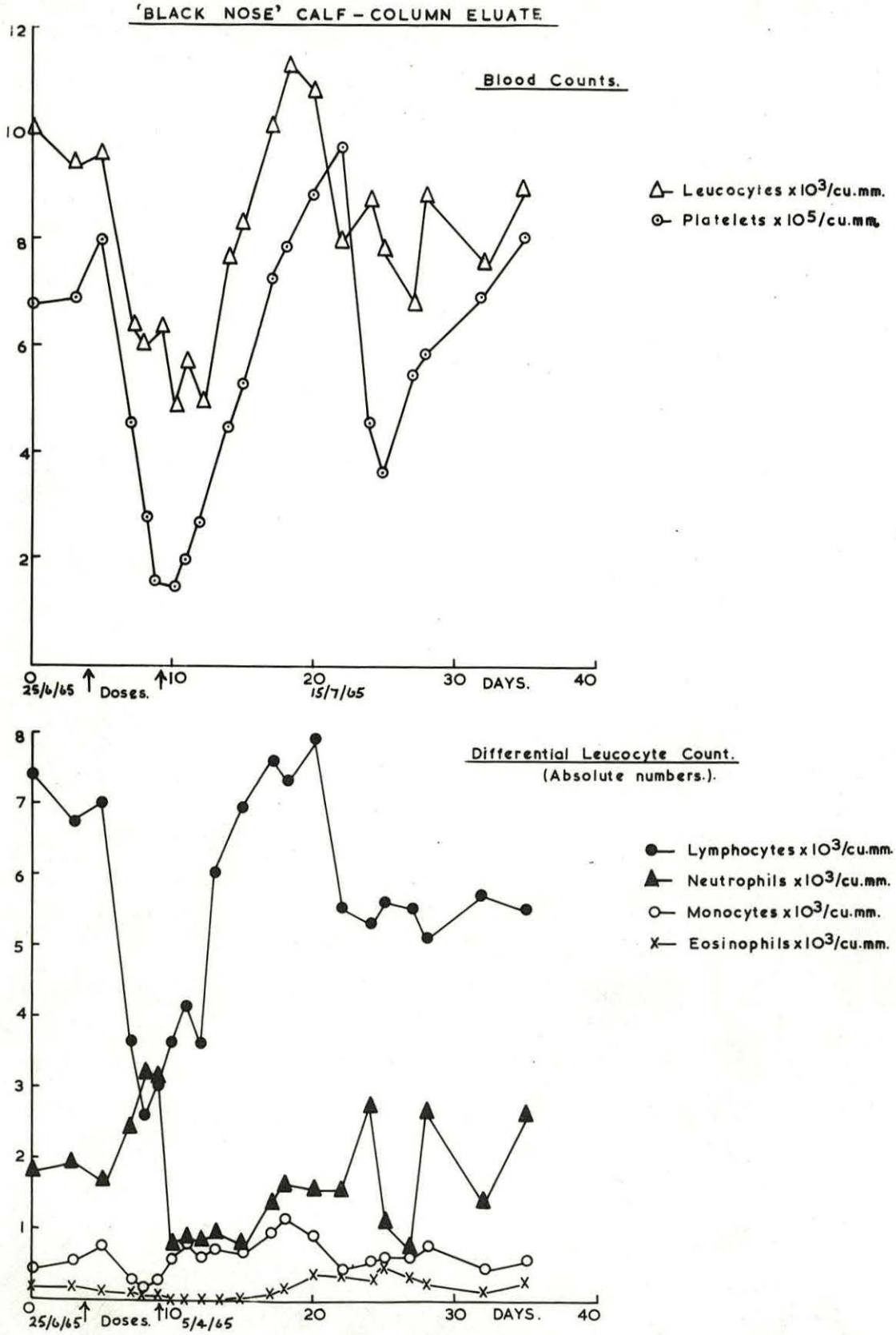

'BLACK NOSE' CALF - COLUMN ELUATE
Blood Counts.
Leucocytes x 10³/cu.mm.
Platelets x 10⁵/cu.mm.
12
10
8
6
4
2
0
25/6/65
Doses.
10
20
15/7/65
30
DAYS.
40
Differential Leucocyte Count.
(Absolute numbers.).
Lymphocytes x 10³/cu.mm.
Neutrophils x 10³/cu.mm.
Monocytes x 10³/cu.mm.
Eosinophils x 10³/cu.mm.
8
7
6
5
4
3
2
1
0
25/6/65
Doses.
10
5/4/65
20
30
DAYS.
40

Fig. 17.

Haemopoietic activity of the Allergic Factor.

The column eluate and the residue were given *per os* to two Friesian calves, weights 80 Kg. and 60 Kg., and aged about six weeks and one month respectively. "Black nose", the 80 Kg. calf, was given 70 mls. of the eluate solution suspended in 500 mls. of water, by drench, on the 29/6/65 and the same amount twice daily from the 30/6/65 to the 3/7/65 and the last dose on the 4/7/65, to give a total of 10 doses.

"White nose", the 60 Kg. calf, was given parallel doses of 110 mls. of the column residue suspended in 500 mls. of water.

The blood counts are shown in Figs.(17 & 18 ). It can be seen that the calf receiving the column eluate showed an almost immediate fall in total leucocytes and platelets, this was accompanied by pyrexia and the animal seemed to be suffering from abdominal pain. Wide-span antibiotics were given at this period as a precaution against infections. The fall in leucocytes was due to a drop in lymphocytes, which was slightly counteracted by a mild transient neutrophilia, giving way in turn to neutropenia.

The other calf "White nose", receiving the residue showed no similar fall in blood cells, the neutrophilia seen may have been related to the animal's scouring. This was possibly due to the change in diet from milk to solid food rather than to an effect of the extract; "White nose" being the younger calf would be more susceptible to chance infections. No pyrexia was observed but wide-span antibiotic therapy was given as a precaution. The scouring stopped after an oral antibiotic was given.

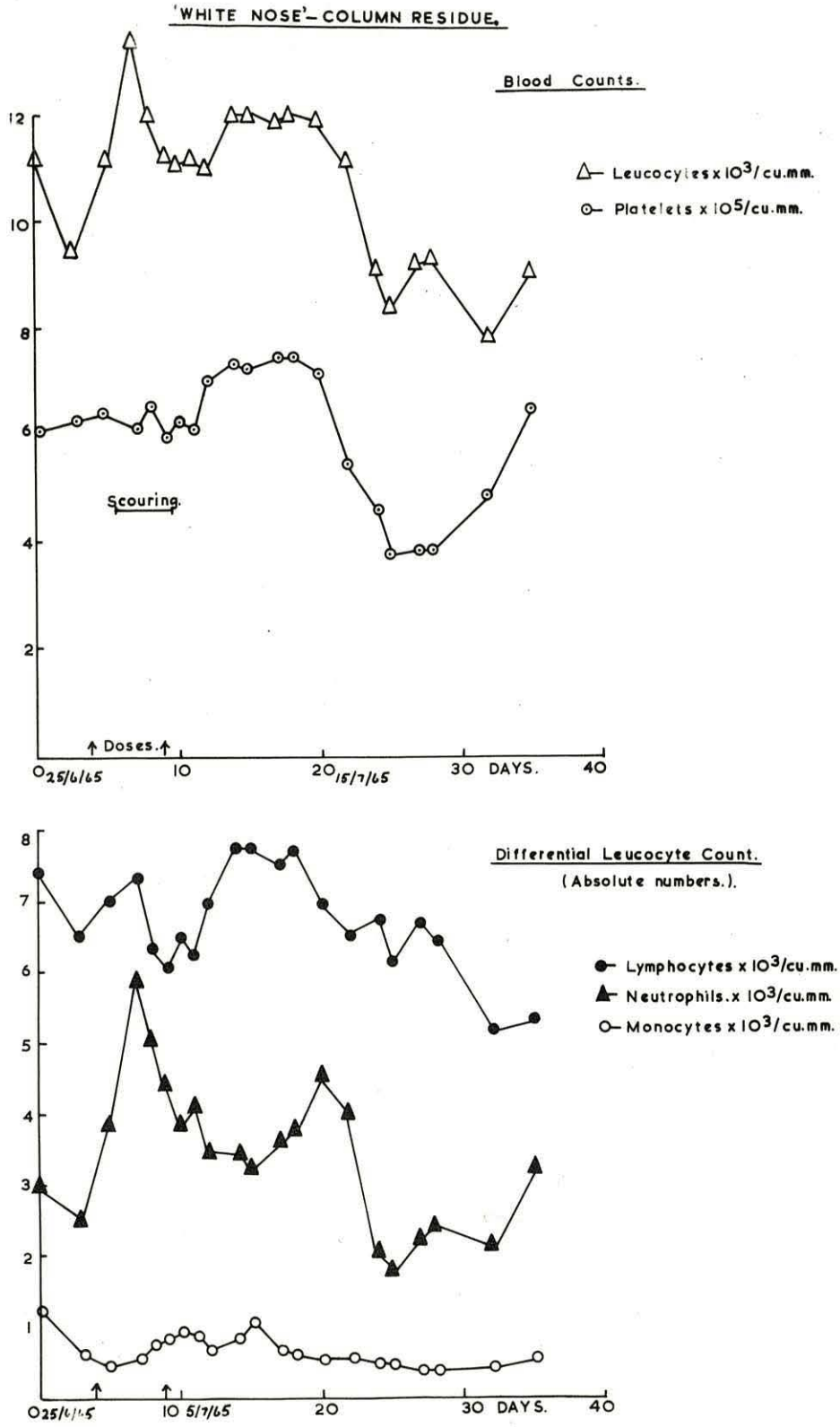

'WHITE NOSE'– COLUMN RESIDUE.
Blood Counts.
Leucocytes x 10³/cu.mm.
Platelets x 10⁵/cu.mm.
Scouring.
Doses.
25/6/65
15/7/65
DAYS.
Differential Leucocyte Count.
(Absolute numbers.).
Lymphocytes x 10³/cu.mm.
Neutrophils. x 10³/cu.mm.
Monocytes x 10³/cu.mm.
5/7/65
DAYS.

Fig 18.

Falls in the leucocytes and platelets of both calves were observed about 20 days following the first dosage, the fall of the "Black nose" occurring after apparent recovery, but the outward appearance of both calves was normal and both animals were gaining weight rapidly.

Differential leucocyte counts showed that the 20 day fall was due to loss of both neutrophils and lymphocytes.

ALLERGIC REACTIVITY OF CALVES TO BRACKEN EXTRACTS.

| CALF. | PREVIOUS TREATMENT. | EFFECT. | TESTED. | REACTION. |
|---|---|---|---|---|
| Anode. Cathode. Neutral. | Fed electrodialysis fractions of aqueous extract from 22/6/64 to 19/7/64. | No effect. | 25/7/64. | Positive.(10*) |
| 6 College Farm animals. | — | — | 29/7/64. | Negative. |
| x Friesian 1964. | Fed bracken 18/7/64. | Died 21/8/64. | 16/8/64. | Negative. |
| Charollais II. | Given extract with no effect 4/2/64–8/2/64. Given 6·5Kg bracken 10–21/8/64. | Platelets 190th., leucocytes 4·6th. neutrophils 2·8% on 9/9/64. | 9/9/64. | Possible.(6?) |
| A Friesian. | Bracken 12 months before. | Slight effect. | 13/8/64. | Possible(6?). |
| same calf. | injection of fractions previous week. | Possible reaction. | 20/8/64. | Positive(8*). |
| Black calf. | Fed acetic acid extract 8/9/64–5/10/64. | No effect. | 16/10/64 | Positive(8*) |
| x 154. x Aberdeen Angus. | Used for split dose effect. | Marked effect | 21/4/65. | Positive(10*) |

x— Case History in this Thesis.

Fig. 19.

Allergic reactivity of calves to bracken extract.

No experiments were exclusively designed for comparative studies, however, some information could perhaps be gained from an analysis of the animals which have been tested during the course of the work.

The histories and reactivity of the various calves used are summarized in the table in Fig.( 19 ).

This limited evidence would seem to indicate that calves having ingested bracken or bracken extracts are hypersensitive to bracken. Small quantities, insufficient to cause a detectable haemopoietic effect, seem to be adequate to sensitize the animal. Calves actually going down with the syndrome have not reacted or reacted only weakly. There are indications that sensitivity to bracken takes at least a week to develop, and does in fact decline over a period of months.

It should be noted that the small amounts given by injection are possibly sufficient to sensitize an animal.

These last observations are substantially similar to findings reported in the TB test.

Discussion.

The delayed hypersensitivity test has shown that calves do become sensitized to factors in bracken after ingesting the crude plant or extracts. It was realised at the outset of this work, that the animals could be sensitized to factors completely unrelated to the haemopoietic factor, since animals are known to become sensitized to their own dietary proteins; however the initial extractions showed that the factor was not a large molecular weight substance and so may have reacted as a Hapten. Another handicap was the fact that animals had to be used repeatedly for tests, and it was possible that the initial tests sensitized the animals to factors not absorbed through the gut, and therefore not concerned with the haemopoietic syndrome. As only a limited supply of animals was available, this could not be avoided, but every effort was made to check results on a fresh animal, to use as many animals as possible and to use them as sparingly as possible.

Calves on extracts, but with no indication of bone marrow damage and consequent fall in circulating cells, were shown nevertheless, to be sensitized. It would be difficult to believe that these extracts contained absolutely no activity with respect to the bovine haemopoietic syndrome. In this context it was shown that an animal gave a positive reaction one week after having had the extract from 3 grams of June 1964 bracken powder in the skin test, so that it would seem that only very small amounts are required to induce sensitivity.

While no worthwhile figure can be given as the lower limit to which a detectable immunological response can normally be evoked, since antigens vary tremendously in their capabilities, amounts of the order of 100-1000 µg. of protein/ Kg. are known to produce responses. Calves fed over several days or weeks would be subjected to the continued presence of antigen, and this is known to increase the response provided the amount is not too high. If the factor was in fact Haptenic in mechanism, then small amounts would be capable of altering large amounts of protein or polysaccharide.

Calves going down rapidly after three weeks on bracken have not shown reactions. The immunological response may not have developed sufficiently during this phase, since the Mantoux test is also negative during the early stages of infection (about 3 weeks); however, the tissue manifestations previously described do indicate that some kind of immunological-inflammatory reaction is taking place. Unfortunately, there was no opportunity to settle this point by testing a bracken-fed calf for reactivity in the period between 7-21 days.

The failure is probably related to the desensitization by excess of the antigenic material, since often during a rapidly progressing infection a negative result occurs with the Mantoux and other bacterial sensitivity tests, because of desensitization with excessive bacterial products. (Humphrey and White 1964). The Russian workers (Klemparskaya et al. 1961) indicate that there is a decrease in allergic reactivity to various antigens in animals presensitized before being irradiated. Irradiation is known to suppress the immunological response and can be used to block tissue homograft rejection, but

these workers suggest that this is not so much a decrease of immunological competence but that the degree of autosensitization is so great as to cause an animal to become refractory to other antigenic stimuli. Dixon and Mc.Conohey (1963) found that irradiation could actually enhance the antibody response, if the antigenic material was given slightly before the irradiation.

It was noticed during the tests, that with the purer extracts the reactions took longer to develop. This could be due to the removal of an adjuvant effect of the pigments, which perhaps provide particulate matter and stimulate phagocytosis.

The immediate decrease in blood cells seen in the "Black nose" animal receiving the eluate have not previously been observed in bracken fed animals. This is possibly because such a concentrated extract, in a presumably available form, has not previously been given; however comparison is not possible for although 9·6 Kgs. was extracted, neither the efficiency of extraction nor the subsequent loss can be estimated. Similarly it is impossible to estimate how much of the haemopoietic factor an animal is normally able to obtain from the powder during the passage through the gut.

It appears that the factor is radiomimetic in so far as it produces the immediate lymphocyte effect in the same manner as alkylating agents (Bacq and Alexander 1961). Workers measuring the effects of irradiation on calves (Rosenfeld 1958 and Schultze et al. 1959) noted similar immediate lymphocyte falls but the platelet falls were more delayed, commencing about 5-6 days after irradiation.

The "Black nose" calf showed pyrexia and intestinal pain during this period, but no scouring analogous to that obtained by Rosenfeld and Schultze et al; it is perhaps possible that the animal was suffering from gastro-intestinal damage analogous to irradiation. This could be responsible for the symptoms since absorption of endo-toxins (SectionII) could cause leucopenia and pyrexia (Humphrey and White 1964). The usual interpretation is that the circulating lymphocytes are directly damaged by the irradiation or radio-mimetic chemicals. It would have been interesting to have taken serial tissue samples from the calf, to investigate the possibility of an inflam-matory invasion of the tissues producing an artificial decrease, however since the platelets also decreased there is a strong possibility of a direct effect.

The "Black nose" calf appeared to show two distinct effects in both platelets and leucocytes, in both cell lines there was an immediate decrease followed by an apparent recovery and then another decrease at about twenty days after the initial dose. "White nose" showed only the second decrease. This twenty day reaction corres-ponds to the effects seen so consistently in previous cases; both animals showed about the same amount of fall although comparison of dose would not be strictly accurate since the "White nose" calf receiving the residue, was twenty Kilos lighter. Since "Black nose" calf apparently received the higher dose, as judged from both skin test and immediate effect, this could indicate that the twenty day effect depended more upon the reaction of the animal than the dose given. Often with immunological reactions the severity and type of reaction depends not on the absolute quantities but on relative amounts.

There is the possibility that two separate factors may be causing the early and late falls, but this is unlikely, although in low doses different classes of alkylating agents may have predominantly one or the other effect (Bacq and Alexander 1961).

The important feature of these experiments is that using an immunological test the radiomimetic factor has been purified to a high degree. The results do not indicate how the immunological mechanisms operate, but that they do operate and are likely to have an important role in the syndrome considered as a whole. It is possible that the substances may form some sort of Hapten combination or they may cause the release of intracellular components not normally circulating, to which the animal becomes sensitized.

The common identity of the allergic factor has not been finally established. However, the elimination of 96% of the ethanol extract with retention of activity common to both would indicate a high probability that they are the same, especially since much of the residual 4% consisted of carotenoid pigments hardly likely to be radiomimetic, and shown not to be allergens.

The validity of the test was further indicated by the fact that the column residue contained the appreciable activity indicated by the skin test.

In conclusion, since the allergic factors and the haemopoietic factors are likely to be the same thing, this is important not only from the point of view of determining the causative agent of bracken poisoning but that the approach indicates the possible importance of auto-sensitization in irradiation disease.

# CHAPTER III.

## THE EFFECT OF DISCONTINUOUS DOSAGE.

## Introduction.

In view of the possible importance of immunological mechanisms in the bracken poisoning syndrome, it was considered that the effects of bracken might be dependent upon the timing of doses rather than directly on the absolute amount given. It seemed unlikely that animals in the field would continuously eat the large amounts of bracken which seem to be required in the laboratory experiments. A more likely possibility would be that the animals ate the bracken at irregular intervals, an animal having eaten bracken might be particularly sensitive to further amounts. In this context France (1964) reported thrombocytopenia and clinical symptoms in a heifer having been in contact with bracken only 4 days. The heifer had been bedded on bracken as a calf.

It is also possible that animals may become resistant and a case has been reported (Carpenter et al. 1950) in which an animal given gradually increasing amounts of bracken did not succumb to further quantities given over a long period, which were sufficient to kill other similar calves quickly.

It was therefore decided to attempt to assess the effects of bracken not given continuously, but in separate amounts separated by an interval in order to assess the importance of pre-sensitization or pre-immunization.

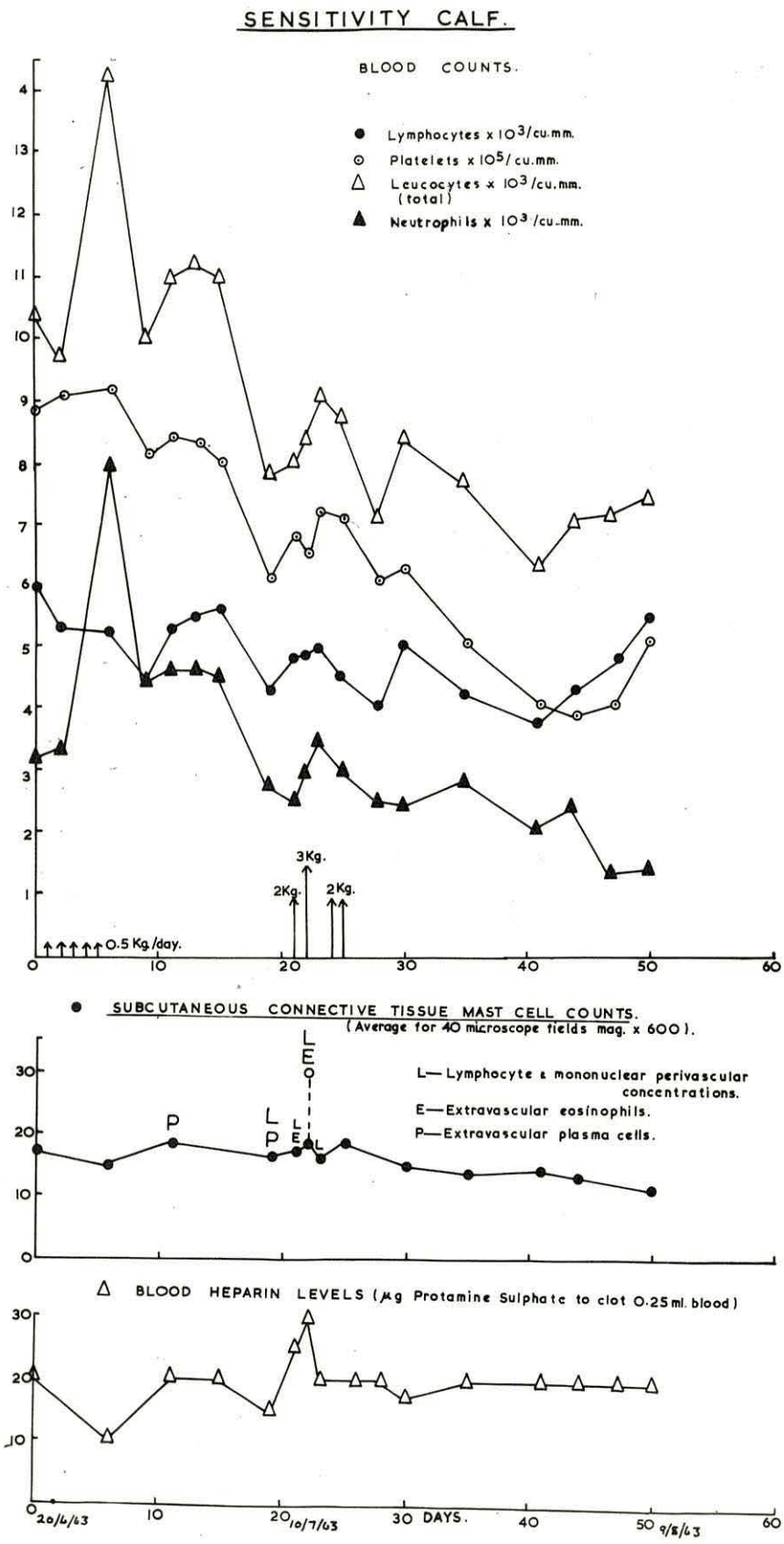

SENSITIVITY CALF.
BLOOD COUNTS.
Lymphocytes x 10³/cu.mm.
Platelets x 10⁵/cu.mm.
Leucocytes x 10³/cu.mm. (total)
Neutrophils x 10³/cu.mm.
0.5 Kg./day.
2Kg.
3Kg.
2Kg.
SUBCUTANEOUS CONNECTIVE TISSUE MAST CELL COUNTS.
(Average for 40 microscope fields mag. x 600).
L— Lymphocyte & mononuclear perivascular concentrations.
E— Extravascular eosinophils.
P— Extravascular plasma cells.
BLOOD HEPARIN LEVELS (µg Protamine Sulphate to clot 0.25 ml. blood)
20/4/63
10/7/63
DAYS.
9/8/63

Fig. 20.

Experimental.

Sensitivity Calf.

The first calf used was given the same rhizome powder as the Red Friesian described previously in this thesis. The rhizome was given in two separate amounts, the first consisting of five daily doses of 500 grams, followed 20 days later by an amount of 9 Kilograms of the rhizome powder given over 5 days.

The effects on the blood counts can be seen from the figure. A drop in circulatory cells occurred 15 days after the first of the 500 gram doses, somewhat earlier than normally seen when continuous dosage is given. The fall was perhaps surprising in view of the fact that 2Kg. / day of the same material given continuously had failed to kill the Red Friesian calf. A small rise and a further slight drop occurred after the second much larger amount. The differential counts revealed a pronounced neutrophilia after the first dosage batch and a second small rise after the larger batch, 20 days later. From the graph it would seem that the animal was about to recover when the second batch was given, however, the counts started to decrease almost immediately. This is something which has not been seen in other animals where a continuous dose was doubled at this stage.

An irregular increase in mast cells occurred on day 22 and was accompanied by a heparinoid release. Although this occurred at the same time as the second dose it cannot be said with certainty that this was due to the second dose, since other tissue manifestations (i.e. the perivascular lymphocyte concentrations and plasma cells) were seen before the second dosage period. The reaction was probably an effec

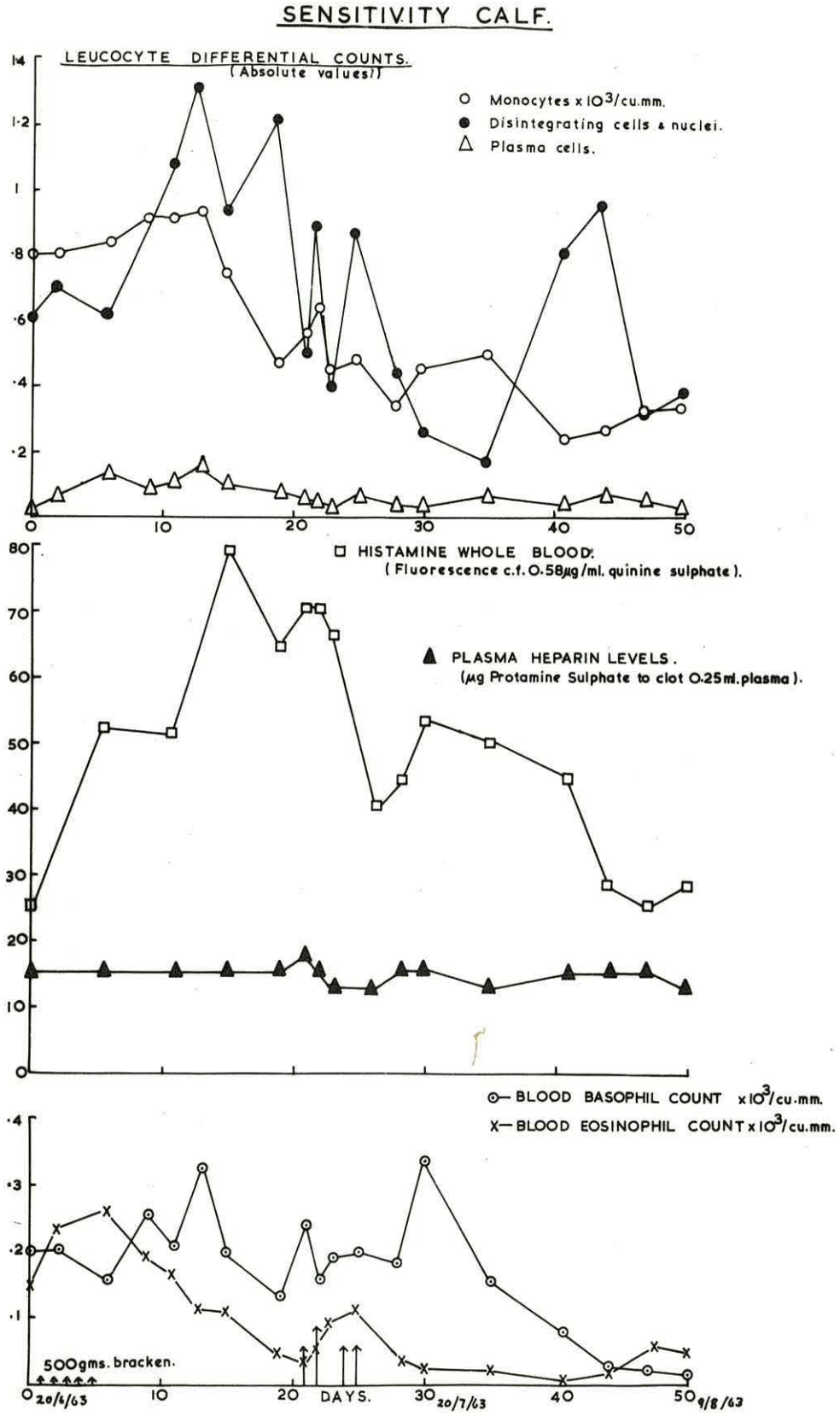

SENSITIVITY CALF.
LEUCOCYTE DIFFERENTIAL COUNTS.
(Absolute values?)
Monocytes x 10³/cu.mm.
Disintegrating cells & nuclei.
Plasma cells.
HISTAMINE WHOLE BLOOD.
(Fluorescence c.f. 0.58µg/ml. quinine sulphate).
PLASMA HEPARIN LEVELS.
(µg Protamine Sulphate to clot 0.25ml. plasma).
BLOOD BASOPHIL COUNT x10³/cu.mm.
BLOOD EOSINOPHIL COUNT x10³/cu.mm.
500gms. bracken.
DAYS.
20/4/63
20/7/63
9/8/63

Fig. 21.

of the first batch of bracken which was coincident with the immediate effects of the second batch.

Disintegrating cells and nuclei were noted during the differential counts and it can be seen that these cells show an increase during the period of circulatory cell fall. The plasma cell count appeared to rise slightly during the period 6-15 days after the initial dose of bracken, possibly indicative of an immunological response.

Unfortunately only whole blood histamine was estimated in this calf, and this seems to follow the circulatory basophil and eosinophil levels indicated. The eosinophils rose sharply immediately after each batch of bracken.

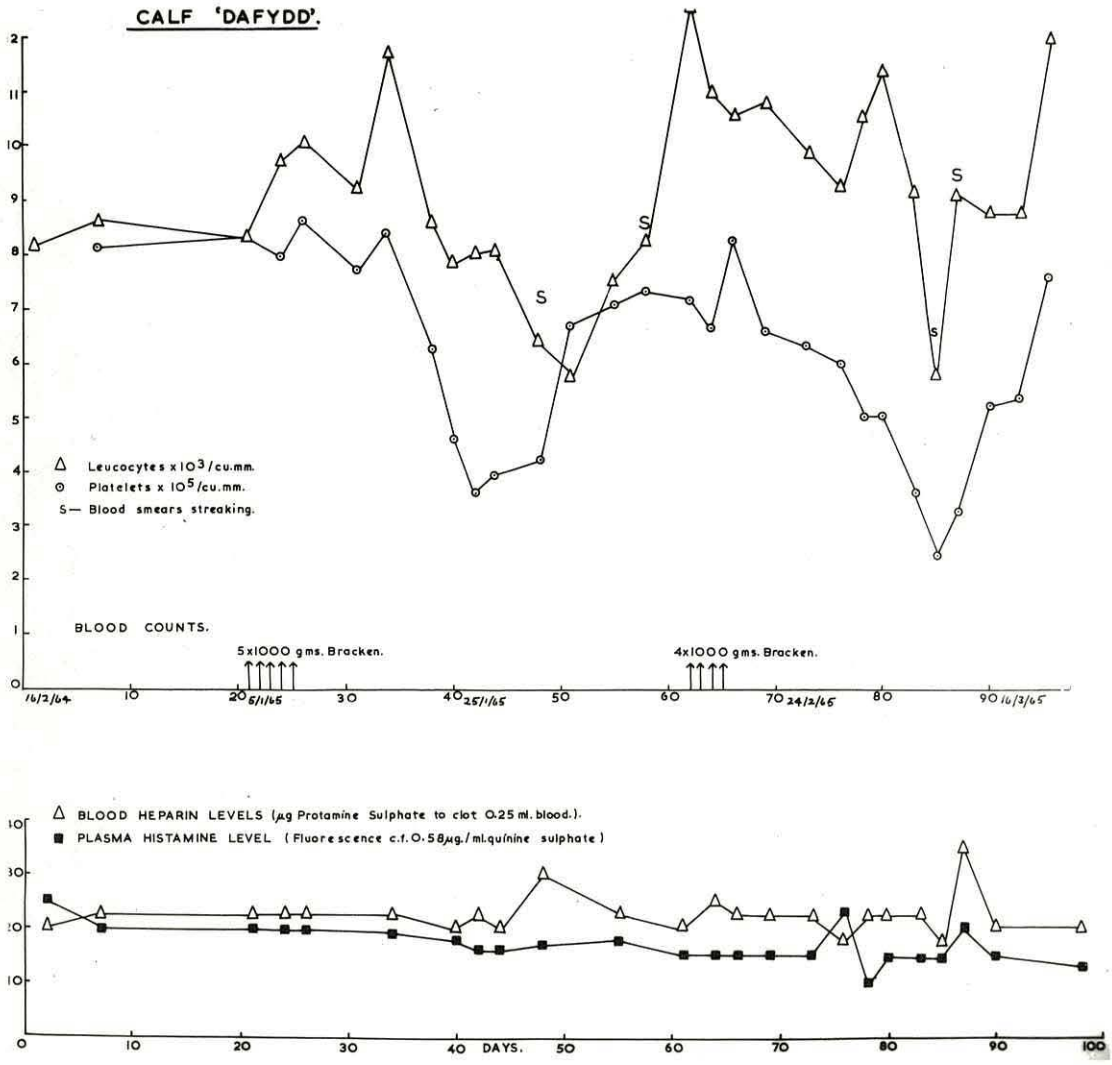

CALF 'DAFYDD'.
Leucocytes x 10³/cu.mm.
Platelets x 10⁵/cu.mm.
S— Blood smears streaking.
BLOOD COUNTS.
5x1000 gms. Bracken.
4x1000 gms. Bracken.
16/2/64
5/1/65
25/1/65
24/2/65
16/3/65
BLOOD HEPARIN LEVELS (µg Protamine Sulphate to clot 0.25 ml. blood.).
PLASMA HISTAMINE LEVEL (Fluorescence c.f. 0.58µg./ml. quinine sulphate)
DAYS.

Fig. 22.

Calves 154 and 'Dafydd'.

These calves were both given the same treatment, with the intention of testing the effects of a pre-sensitizi dose upon subsequent reactivity to bracken. The calves were given 1000grams/day of the June 1964 bracken for five days (154 received an accidental extra ½ Kg.). 41 days later the calves were given 1000grams/day for 4 days. The interva between the batches was made longer than in the case of the sensitivity calf, to allow the calves to recover from the initial dose. This would allow the effects of the second dose to be assessed more clearly.

Calf 'Dafydd'.

The first group of doses produced a marked effect in both leucocytes and platelets occurring 14 days after the first dose, a briefer period than usually seen after continuous feeding. The second group of doses also produced a marked effect, possibly slightly greater, although the amount of bracken given was smaller. The drop in platelets also seemed to start almost immediately after the dosage, following an initial small transient rise. The appearance of streaking on the differential smears was accompanied by the appearance of circulatory heparin on day 87.

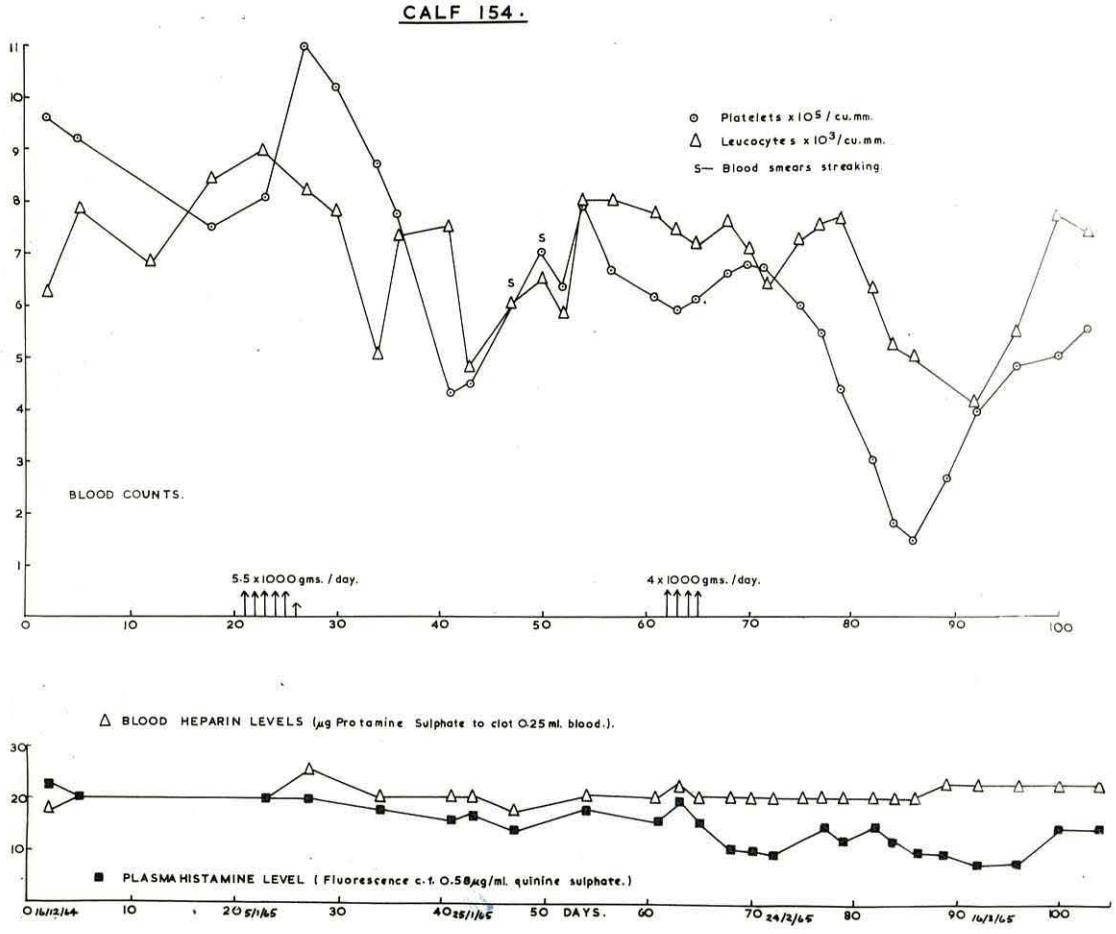

CALF 154.
Platelets x 10^5 / cu.mm.
Leucocytes x 10^3/cu.mm.
S— Blood smears streaking
BLOOD COUNTS.
5.5 x 1000 gms. / day.
4 x 1000 gms. / day.
BLOOD HEPARIN LEVELS (µg Protamine Sulphate to clot 0.25 ml. blood.).
PLASMA HISTAMINE LEVEL ( Fluorescence c.f. 0.58µg/ml. quinine sulphate.)
DAYS.
16/12/64
5/1/65
25/1/65
24/2/65
16/3/65

Fig. 23.

Calf 154.

Results very similar to calf 'Dafydd' were obtained. The second fall in platelets commenced earlier and was more marked than the first fall. No changes in heparin or histami were observed.

Both calves gave positive skin tests to ethanol extracts of bracken on day 61, just before the second batches were given. Neither showed any external symptoms at any time during the experiment, and after the recovery of the blood counts both have remained apparently normal. Unfortunately at the time of writing this thesis the tissue samples and the differential counts which might have revealed important changes were not available.

Discussion.

It is difficult to assess the effects of irregular as opposed to continuous ingestion from the evidence given. It would possibly appear that in the sensitivity calf the effect of the second doses was much less than the first although only 20 days separated the two.

The last two calves possibly showed a greater sensitivity to the second batch and presumably since 40 days separated the two the bone marrow would be fully recovered. It would seem from the results that a few days feeding produced a quicker and more marked effect than would be expected by comparison with the effects produced by continuous feeding at the same rate. Possibly with the latter, the immunological response may be paralyzed.

The consequences of an immunological response, whether leading to greater sensitivity or to greater resistance cannot be assessed from the results, since no really clear cut evidence was obtained. Possibly if the bracken factor formed hapten-like combinations with, for example, proteins it would do so on a non-selective basis with the susceptible sites on all proteins, but the radiation simulating effects would probably be a result of action against specific proteins of particular importance in cell replacement. If, for example, the animal acquired circulating antibodies against the factor plus relatively unimportant plasma proteins, it is possible that the factor might be removed before it reached the important sites. This type of mechanism would possibly explain the early but yet less pronounced second reaction of the sensitivity calf, the animal may have been sensitized and ready to react immediately against these important sites, but also possessing mechanisms to clear the factor before it reached these sites.

The calves 152 and 'Dafydd' may have retained sensitivity but after 41 days little circulatory antibodies.

All this is purely hypothetical and on the evidence available nothing can be concluded. Russian workers (Klemparskaya et al. 1961) noted that resistance to repeated irradiation increased with the development of antibodies, yet stressed the importance of autosensitization in the development of irradiation sickness. Probably sensitization to different body components has different end results and indeed different types of sensitivity i.e. delayed compared with antibody mediated sensitivity has different effects. The sensitivity mechanisms may be important with respect to the tissue manifestations but of less importance with regard to the bone marrow damage.

# SECTION II.

## THE GASTRO-INTESTINAL SYNDROME.

## Introduction.

The possibility of a bracken poisoned animal suffering from the effects of a gastro-intestinal syndrome analogous to that caused by irradiation was considered; especially in cases where despite bone marrow recovery, absence of massive haemorrhages and reduction of pyrexia there is a fatal termination. (Evans I.A. 1963). Some of the animals lose weight during the final stages and most appear to suffer intestinal pain and occasionally marked tenesmus. Blood and mucus are observed in the faeces (Evans W.C. *et al.* 1954), and *Post mortem* examination usually reveals widespread petechial haemorrhages throughout the intestinal tract. Naftalin and Cushnie (1954) noted that the intestinal submucosa and subserosa were often oedematous and some areas were infiltrated with mononuclear and plasma cells; ulceration was frequent. Animals not given antibiotic therapy are very prone to bacterial infections of intestinal origin and it is possible that most untreated field cases die eventually from bacteraemia.

In view of this evidence it was thought worthwhile to examine the possibility of intestinal damage since this might have considerable influence on the nature of the therapy required.

## The gastro-intestinal syndrome in irradiation.

Studies of radiation dose-survival relationships in several species of laboratory animal have led to the description of a specific lethal syndrome, characterized by death after 3-5 days and associated specifically with exposure of the intestinal tract (Quastler 1956). Anorexia,

nausea, vomiting and copious, often sanguinous diarrhoea, frequently develops.

Histological.

The morbid anatomy consists predominantly of epithelial damage of the small intestine (Montagna and Wilson 1955, Lesher 1957). Willoughby (1961) described the changes in rats subjected to 1050r over the intestine. Sections of the intestine at 24 hours postirradiation revealed oedema, lifting of the epithelium from the connective tissue, gross shrinkage of the villi and flattening of the epithelial cells; after 48 hours all these effects were enhanced and dilation of the central vessels of the villi with the appearance of fibrinous material indicated haemostasis. The intestine showed many signs of regeneration by the fourth day, mitotic activity becoming evident and vasodilation and oedema decreased. Mitoses became numerous by the fifth day and appeared normal. Conard et al.1956 describe similar events in dogs.

The syndrome may be described as a cessation of mitotic activity by the epithelial cells lining the bottom of the crypts. This causes a reduction in the height of the villi as the normal upward movement of the cells fails to replace those lost by desquamation, leading inevitably to a loss in surface area and presumably of functional capacity. At its most severe this may lead to a denudation of the mucosa over wide stretches.

Electrolyte excretion.

The mean sodium loss of rats given 1500r of X rays was found to be 1-5 m.equivalents in excess of fasting controls, (Jackson, Rhodes and Entenman 1958) this loss occurred chiefly on the third day after irradiation prior to death on the fourth day, and by way of the intestinal tract. The removal of an equivalent amount, but not of less than 1•26 m.equivalents, by intraperitoneal dialysis with hypertonic glucose solution in fasting non-irradiated rats caused death within 24 hours. It was concluded from these results that the loss was the immediate cause of death in the rats.

Studies by Jackson and Entenman (1959) indicate the importance of bile secretion in the syndrome in rats (1500r). Ligation of the bile duct increased the mean survival time to 5•6 days compared with 3•3 days for sham-operated controls. They conclude however, because diarrhoea eventually developed in irradiated rats subjected to the bile duct ligation, that leakage of fluid and electrolytes directly across the intestinal mucosa may become an important factor when treatment which prolongs the survival time is given.

Sullivan (1962) found that diarrhoea did not develop in rats subjected to 1000r X rays and having biliary flow interrupted by ligation or cannulation. Diarrhoea did however develop in irradiated cannulated rats and in unoperated controls when bile or bile salts comparable to the normal daily output were injected into the duodenum. He concluded that in some way bile salts are involved in the development of radiation diarrhoea, and

that the diarrhoea is not due merely to a radiation-induced defect in the cellular turnover of intestinal epithelium.

However, his results appear to conflict with those of Jackson and Entenman (1959) who found that diarrhoea did eventually develop in rats subjected to bile duct ligation.

Effects of sodium depletion.

Sodium is found in mammalian tissues as the chief extra-cellular ion, it is thus concerned with the maintenance of the pH of the body fluid. Studies in the osmotic behaviour of cells (Conway 1957) indicate its function as the primary regulator of body fluid osmolarity and consequently in the stabilization of the body fluid volume. The realization that sodium (and water) depletion in such diseases as infant diarrhoea and Asiatic Cholera could lead to clinical symptoms, was responsible for the development of parenteral fluid therapy (Marriott 1923).

Elkington and Danowski (1955) have summarized the work on the effects of sodium depletion. In the experimental animal, salt depletion leads to a decrease in both plasma and interstitial fluid volume; circulation time and the arterio-venous oxygen difference increase, and there is a concomitant fall in the cardiac output and arterial pressure. The elasticity of subcutaneous tissues decreases and impairment of renal function leads to a rise in blood urea. A state of shock results, but often in such situations death can be prevented by parenteral administration of sodium salts.

Bovine Bile Secretion.

The seriousness of the gastro-intestinal syndrome in the calf would seem to depend on the rate of loss of sodium through the bile. Nichols and Nichols (1956) show that the loss of more than 20% of the body sodium would have very serious consequences for an animal. Jackson and Entenman (1959) calculated the approximate length of time it would take for several species to secrete 20% of their body sodium at normal rates of bile secretion. These show values of 4-6 days for the larger animals (Cat 4•8, dog 5•6, Sheep 5•5, Man 4•2) compared with ½-2 days for the smaller laboratory animals.

A search through the literature for calf bile flow rates has proved fruitless, however Schmidt and Ivy (1937) obtained very similar figures for bile pigment flow rates/ Kg. body weights of adult sheep, goats and bovines. If a tentative comparison is drawn between the large animals of Jackson and Entenman (1959) and the bovine, it would seem that the sodium loss through this channel would be a relatively slow process.

The bovine has another advantage in that the normal animal possesses the ability to absorb sodium across a considerable concentration gradient in the lower part of the small intestine and the large intestine, so that relatively little is lost in the faeces when the animal is subject to sodium depletion. (Renkema et al. 1962). As the large intestine would possibly suffer less damage than the small intestine, the bovine could still have a high ability to retain sodium after radiomimetic injury.

The gastro-intestinal syndrome in the bovine.

Schultze et al.(1959) has described the appearance of diarrhoea in calves receiving 250-350r of whole body gamma irradiation, the diarrhoea became obvious 3 days post-irradiation and subsided after a further two days. Most of the animals died from 14 to 24 days after irradiation from the effects of the haemopoietic syndrome and during the last 2-3 days blood and mucous were frequently seen in the faeces. One animal receiving 350r behaved exceptionally in that the diarrhoea became progressively worse and the calf died on the fifth post -irradiation day.

Rosenfeld (1958) observed the immediate transient appearance of diarrhoea following 600r. whole body gamma irradiation of 100-130 Kg. calves. After a phase of apparent well-being lasting 5-6 days post-irradiation this reappeared and probably played an important part in the deaths which occurred, although Rosenfeld describes the reappearance of mitoses within 72 hours and eventual complete repair of the epithelium. The mean survival time of the animals used was 10 days.

It is possible that calves receiving continuous doses of bracken might suffer acute damage and consequent salt loss as well as the more chronic effects considered as part of the haemopoietic syndrome.

Further Incidental Effects of Gut Damage.

Endotoxins.and Cell Breakdown Products.

Taketa (1962) suggested on the basis of his results with antibiotic therapy, that toxaemia due to bacterial

endotoxins, possibly emanating from the intestinal flora, must be considered as a possible factor promoting death. Ravin and Fine (1962) concluded that the rate of absorption of endotoxin from the intestine is determined by a number of variable factors. The effects of cell damage and layer denudation would increase this, then also the effect of impairment of the reticulo-endothelial system by the deficient blood flow after irradiation and direct damage would mean that the endotoxin would remain free to exert its effects on the host tissues.

Willoughby (1961) found that the anti-esterase D.F.P.(Di-isopropylflurophosphonate) reduced mortality in rats after 1050r X irradiation apparently by its anti-inflammatory action on radiation injury to the intestine. He suggests that by suppressing the vascular changes following injury, endotoxaemia from the intestinal flora is prevented, or if D.F.P. affects cellular permeability the leakage of toxic substances from necrotic cells might be diminished.

Russian workers suggest that the destruction of the cellular elements of the intestine has a very important role in the aetiology of irradiation sickness (Klemparskaya et al.1961), and that by enhancing tissue breakdown the micro-organisms increased the effect. The development of auto-sensitization was followed extensively by these authors and they were of the opinion that auto-sensitivity to the intestine and intestinal contents played a fundamental role in irradiation sickness. They were able to show that presensitization with homologous intestinal tissue increased the fatality rate after irradiation. The mitochondrial fraction in particular was highly toxic when injected into unsensitized animals, producing pyrexia,

leucopenia, and haemorrhages in the lungs and intestinal tract.

The role of auto-sensitization in irradiation and bracken poisoning was discussed in previous chapters.

Approaches to Therapy.

Antibiotics.

Conard et al.(1956) working with irradiated dogs gave parenteral infusions of balanced electrolyte solutions, plasma, protein hydrolysates, anti-biotics and vitamin supplements, but Taketa (1962) working with rats suggested that micro-organisms may play a more prominent role than dehydration, but that dehydration must be taken into account, he obtained the best survival times from a combination of NaCl and antibiotic therapy. Byron et al.(1964) concluded that an antibiotic regime prevented the appearance of anorexia, diarrhoea and electrolyte loss in 820r X irradiated monkeys.

Evans et al.(1958) have stressed the importance of antibiotic therapy in cases of bracken poisoning and from the above results and observations of gastro-intestinal bacterial invasions in bracken poisoned calves it would seem evident that widespan antibiotic therapy is essential.

Saline.

Therapy in cases of bovine bracken poisoning would depend largely on whether any intestinal damage caused sodium loss, or simply an impairment of absorption of nutrients. If acute sodium loss did occur, it would

be necessary to replace the loss by parenteral injections of physiological saline; if however, acute bile mediated losses do not occur, but the absorptive function is impaired, more comprehensive therapy analogous to that of Conard et al.(1956) could be tried.

Aldosterone.

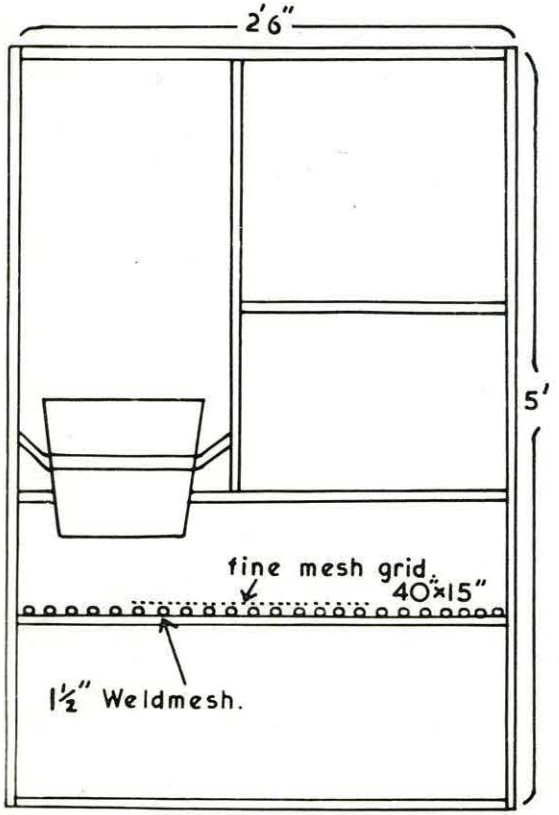
The possibility of mineralocorticoid therapy was considered in view of the importance of sodium retention in irradiation.

There are three sites where mineralo-corticoids exert their action, kidney, skin and intestine. Of these the kidney is the most important and direct injection produces an immediate increase in tubular reabsorption (Barger et al. 1958). The concentration in sweat appears to be inversely proportional to adrenocortical function (Conn 1949) while Dennis and Wood (1940) found decreased sodium absorption from the ileal loops of adrenalectomized dogs.

Aldosterone therapy would thus seem likely to increase the sodium retention of bracken poisoned calves, possibly faecal loss could be reduced since Bott et al. (1964) working with cattle concluded that faecal conservatio was determined by aldosterone, this could be important since any loss is likely to be by this route.

Extra-cellular potassium if raised above the normal level can be toxic (Comar and Bronner 1962) and some of the manifestations of this are part of the central nervous convulsive syndrome, Rats receiving less massive doses of irradiation than those producing this immediate syndrome show increased urine potassium levels indicative

METABOLISM CRATE.




END ELEVATION.

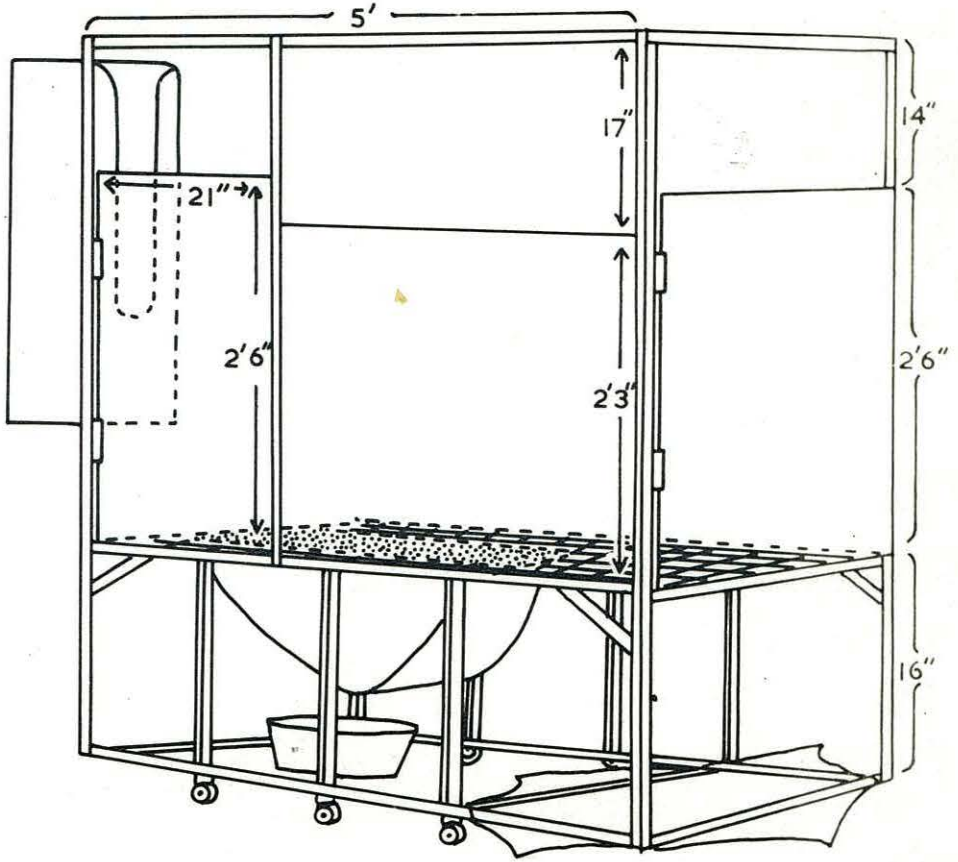



Fig. 24.

of cellular damage(Jackson, Rhodes and Entenman 1958). Since aldosterone therapy increases preferentially the excretion of potassium (Gaunt, Renzi and Chart 1955) the drug would possibly be of value in eliminating potassium released from damaged cells.

Aldosterone mould also have another useful function in that it appears to have a prophylactic action against the effects of bacterial toxins (Bein and Jaques 1960) which may be absorbed through the intestine.

Aims.

In view of the evidence it was decided to attempt to assess the possible involvement of a gastro-intestinal syndrome in bracken poisoning, and resultant upon the findings to plan possible therapy.

Most species contain about 65m.equivalents of sodium/Kg. fat free tissue (Comar and Bronner 1964); so that a 135 Kg. calf(Total body content 180 grams) would have to lose 35-40 grams of sodium to be in serious difficulties. Cellular damage could possibly be indicated by potassium loss in the urine (Jackson, Rhodes and Entenman 1958).

It was thought therefore, that a total balance experiment of sodium and potassium would supply information about a possible loss of intestinal function and consequent therapeutic needs, and examination of the section of intestine would reveal the degree of intestinal damage and thus indicate its importance in the haemopoietic syndrome.

# EXPERIMENTAL METHODS.

## Calf Crate.

An initial attempt was made to collect urine and faeces using bags attached to the animal, but it was found that this method was not reliable over the long periods required for the experiments.

It was decided that a method which was more comfortable for the animal and more dependable during the terminal stages when the animal is *in extremis*, would be to enclose the animal in a crate. Such a construction, represented in Fig.( 24 ) was developed to be suitable for 100-150 Kg. calves.

The crate was constructed mainly from aluminium, the framework being made from "Dexion" and the sides from sheets of aluminium, with a floor of galvanized "Weldmesh" ( Commercial Product). The doors at the front provided easy access for bleeding from the jugular vein, without removing the animal from the crate; this is important because of the possibility of a "physiological leucocytosis" following any major disturbance. The low sides enable tissue samples to be taken easily. The animal was tethered to the front of the crate, and since only male calves were used, it was possible to collect the urine by suspending a collection sheet of polythene leading to a container; and to allow the faeces to fall through the Weldmesh floor to a subjacent tray. It was found that very little intercontamination took place if the polythene sheet was positioned properly. The calf had three restricting influences which determined his relative position, his front tether, a bar placed

across behind his haunches and a finer mesh placed on the Weldmesh to a distance of 40" back from the front. The calves found this preferable to stand on although they did not appear to find the Weldmesh, (normally used for this purpose agriculturally) uncomfortable.

The little contamination which did occur was due to faeces in the urine which did not affect the results greatly because of the higher urine salt content. This contamination was never more than of the order of grams and was partially eliminated by filtration of the urine samples. A food container of the dimensions shown was used and the high sides allowed only negligible wastage.

Diet.

The first attempt at a sodium and potassium balance experiment involved an animal being fed a normal diet of hay and proprietary brands of cattle cake, and the great difficulty encountered in estimating the intake was due in part, to the large variations between hay samples and partly to the unpalatability and consequent variation in the consumption of the bracken powder.

When facilities for pelleting became available, it was found convenient to feed the animals solely on a composite ration of pellets consisting of 66% cattle cake and 33% dried bracken frond by weight, which was both nutritionally adequate and very palatable.

Preparation of the composite diet.

Bracken Powder.

Variations in the bracken samples have previously made comparative experiments very difficult, therefore in June 1964 a month was spent preparing enough of the dried

plant to provide material for a year's experiments. The following technique was evolved.

Bracken frond was gathered from a roadside site in the Nant Ffrancon valley by hand-stripping the fronds from the mainstem and this material was then tumbler-dried for 60 minutes; the temperature of the effluent air current at the end of this time being 50°C. After this processing the fine leaf material had fallen through the tumbler dryer mesh into the trays, and the remaining coarse material was rejected. The fine material was oven dried at 40°C for 48 hours and then milled.

The entire month's collection in the form of a fine powder was then bulked and mixed so that a large quantity of homogeneous material was available. Subsequent experiments showed that this material was highly active and that 1000g / day was sufficient to kill a 150 Kg. calf in about 30 days.

Composite diet preparation.

25 Kg. of cattle cake was milled and mixed with 12•5 Kg. of the bracken frond powder in a mixer (Apex 255C) for 20 minutes; after addition of 3•75 litres of water and a further 20 minutes mixing, the mixture was pelleted. Most of the water was lost during the pelleting process where some heat is generated and the pellets were allowed to dry out. 3 Kg. of the diet / day was normally fed to a 125 Kg. calf.

Estimation of Sodium and Potassium.

Faeces.

A polythene sheet was sited on the tray under the

floor of the crate to catch the faeces which fell through the Weldmesh, any residue was collected by brushing with a stiff long bristled brush. The entire sample was weighed, then mixed with a trowel on the polythene sheet and a representative sample of approximately 300 grams was obtained by the method of quartering. This was stored in a closed polythene bag in a cold room until required.

For analysis three samples of approximately 20-30 grams were taken and weighed in 400 ml. tall, lipless beakers. Initially the wet ashing method of Luna (1959) was used, but subsequently it was found sufficient to add 150 mls. of conc. nitric acid to the samples. The beakers were then covered with clock glasses, heated gently until thick brown fumes had ceased to come off, and then heated strongly for several hours until the solution was clear and the volume reduced to approximately 15 mls. The ready solubility of sodium and potassium salts permit quantitative release by this method.

The samples were then diluted to 100 mls. with distilled water and aliquots of 25 mls. taken which were diluted to 500 or 1000 mls. This gave final concentrations of less than 10 p.p.m. sodium and 20 p.p.m. potassium. The solutions were estimated directly on a Zeiss Flame Photometer at wavelengths of 560 mμ and 768 mμ respectively, by comparison with standards of 10 p.p.m. and 20 p.p.m., At these particular levels the relationship between the instrument reading and the concentration is linear.

The following table indicates the usual variation between samples.

FRIESIAN 1964. Final 4 days. ( 17/8/64 - 20/8/64.).

| | | $Na^+$ | | | | $K^+$ | | | |
|---|---|---|---|---|---|---|---|---|---|
| Total wt. (gms) | Sample (gms) | ppm. | mg. /gm. | mean | Total | ppm. | mg. /gm. | mean | To |
| 2100 | 23•663<br>26•138<br>17•280 | 2•15<br>2•5<br>1•7 | 0•364<br>0•383<br>0•394 | 0•380 | 0•80 | 22•5<br>25•5<br>16•5 | 3•8<br>3•89<br>3•84 | 3•84 | 8•( |
| 2000 | 19•360<br>27•344<br>23•792 | 2•8<br>3•8<br>3•4 | 0•578<br>0•556<br>0•572 | 0•569 | 1•14 | 15•5<br>22•5<br>19•5 | 3•2<br>3•29<br>3•27 | 3•25 | 6•! |
| 1350 | 25•00<br>25•00<br>24•5 | 6•4<br>6•4<br>6•1 | 1•024<br>1•024<br>0•995 | 1•01 | 1•36 | 19•0<br>19•0<br>18•5 | 3•04<br>3•04<br>3•02 | 3•03 | 4• |
| 950 | 19•530<br>23•612<br>24•582 | 4•3<br>5•1<br>5•2 | 0•880<br>0•865<br>0•847 | 0•864 | 0•82 | 15•0<br>19•0<br>19•5 | 3•07<br>3•22<br>3•18 | 3•16 | 3•( |

The average variation between samples from the mean was 1•85% for sodium and 1•1% for potassium, which is not much more than the accuracy of the flame photometer.

Urine.

The urine was collected in a polythene sheet which drained into a container, a 24 hour sample was collected, the volume measured and a 300-400 mls. aliquot taken. This sample was immediately filtered through coarse grade filter paper, rejecting the first third of the filtrate 20 mls. of the filtrate were taken and diluted to 100 mls. with distilled water. 25 mls. of this diluted to 250 mls., and 25 mls of this solution diluted to 250 mls. gave a solution which was read directly on the flame photometer.

Diet.

Samples of approximately 6 grams were taken and wet ashed by the same method as that used for the faeces and diluted in the same manner.

When the animals were being fed on a normal mixed diet this proved to give the most inaccurate estimations because of sampling errors. The compound diets eventually used and the proprietary cattle cakes showed only slight variations within a batch, but the hay samples showed great variations which chaffing and thorough mixing only partially eliminated. The 1964 compound diet showed the following variations between samples:

4•46, 4•4, 4•28, 4•25, 4•34, 4•45, 4•46. (Mg.$Na^+$/ gm).
14•15, 13•9, 13•85, 14•15, 14•2, 14•3, 13•95. (Mg.$K^+$ / gm).

Histology.

Specimens from all parts of the gastro-intestinal tract were taken as soon as possible after death, this was usually within two hours, so that any changes observed would not be due to post mortem deterioration. The samples ($\frac{1}{2}$ x $\frac{1}{2}$ inch) were kept flat during fixation by cutting the intestine open on a piece of filter paper, so that the lumen part of the intestine and consequently the villi were uppermost. The fixation was carried out in 80% ethanol for at least 2-3 days.

For sectioning, a small piece of this was cut off and embedded with Gurr's Paramat wax using 2-Ethoxy-Ethanol as a solvent. Transverse sections of 7μ were normally cut and the wax cleared with Xylene. After transferring to ethanol the sections were stained with Wright's stain by the method described for the tissue sections.

# EXPERIMENTAL.

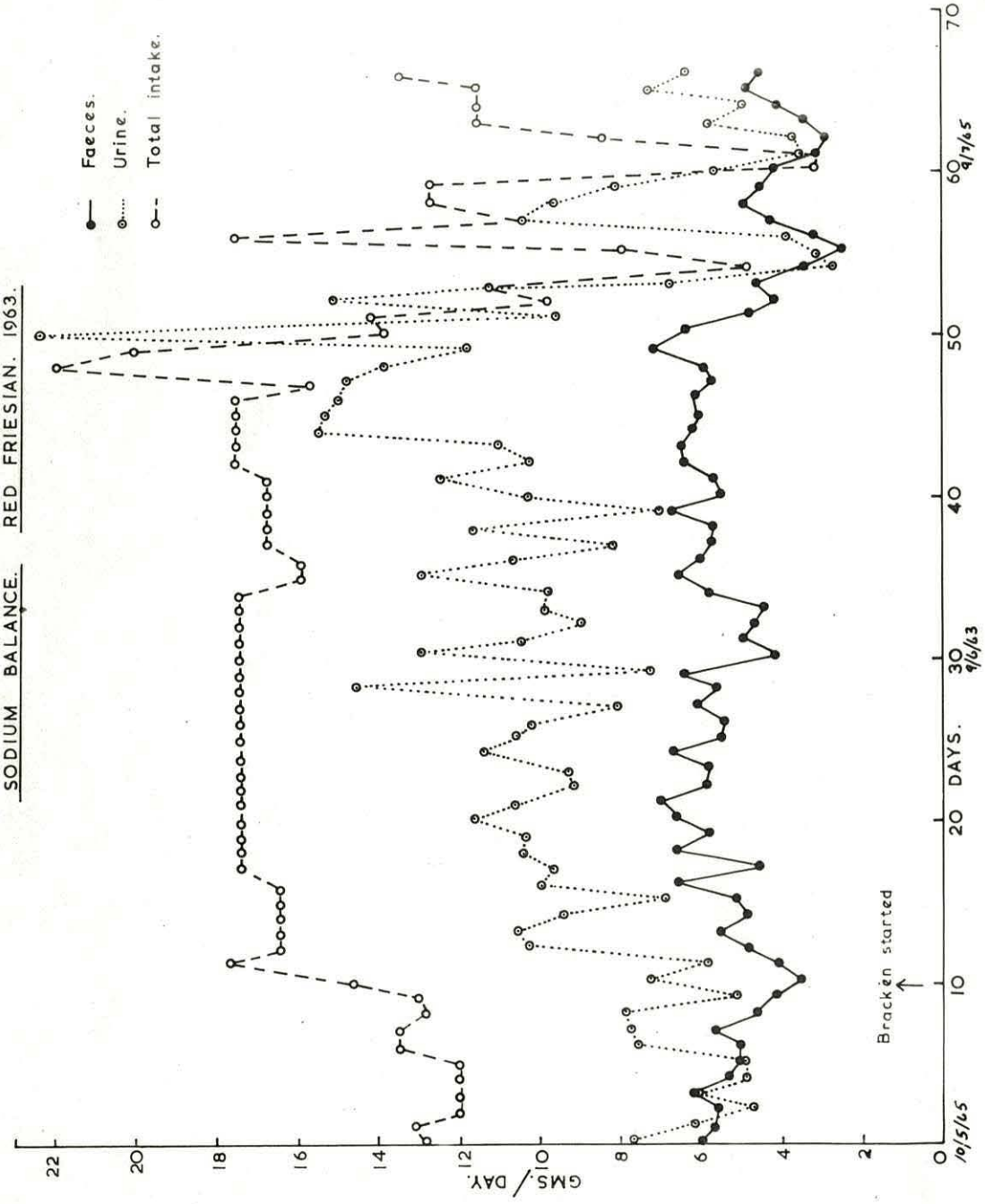

SODIUM BALANCE. RED FRIESIAN. 1963.
Faeces.
Urine.
Total intake.
GMS./DAY.
DAYS.
Bracken started
10/5/63
9/6/63
9/7/63

Fig. 25.

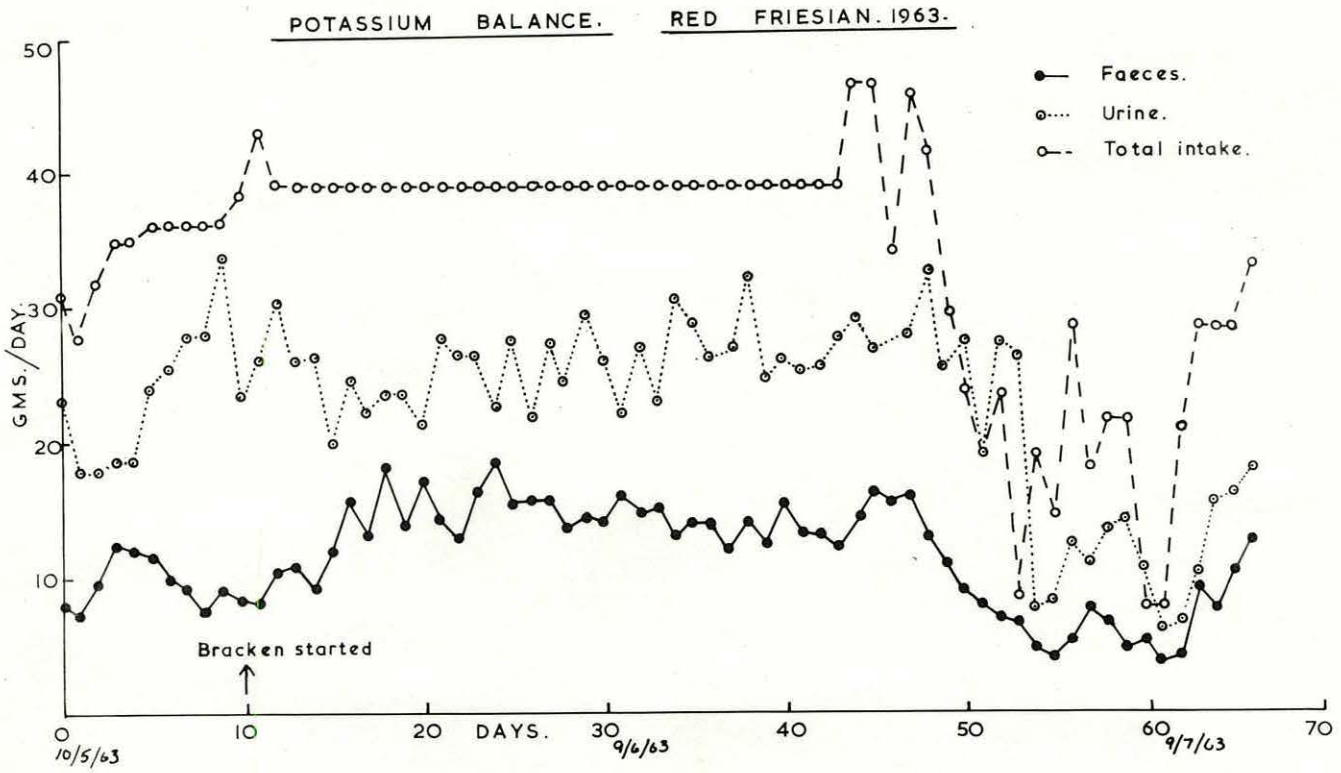

POTASSIUM BALANCE. RED FRIESIAN. 1963.
Faeces.
Urine.
Total intake.
GMS./DAY.
50
40
30
20
10
Bracken started
0
10/5/63
10
20
DAYS.
30
9/6/63
40
50
60
9/7/63
70

Fig 26.

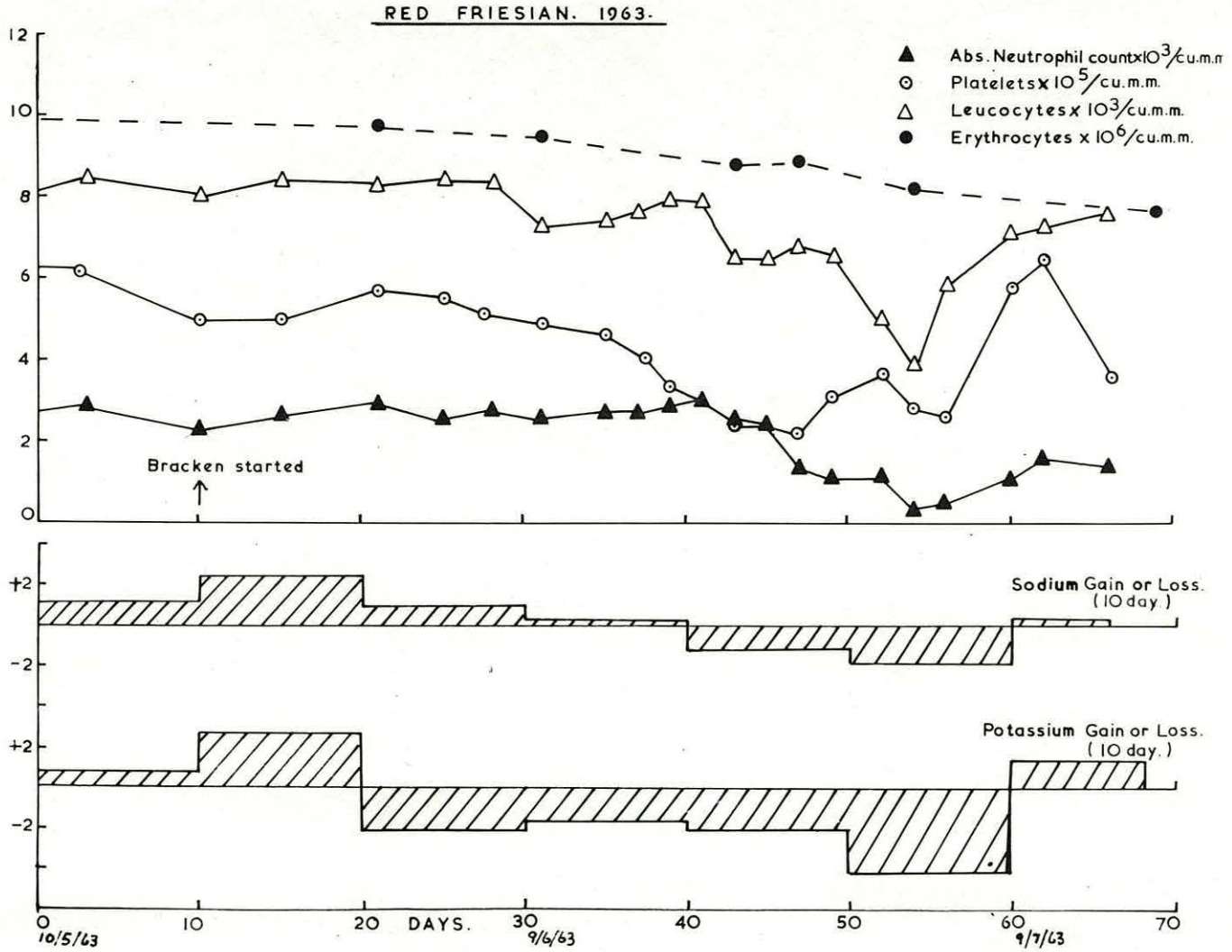

RED FRIESIAN. 1963.
Abs. Neutrophil count x 10³/cu.m.m
Platelets x 10⁵/cu.m.m.
Leucocytes x 10³/cu.m.m.
Erythrocytes x 10⁶/cu.m.m.
Bracken started
Sodium Gain or Loss. (10 day.)
Potassium Gain or Loss. (10 day.)
DAYS.
10/5/63
9/6/63
9/7/63

Fig 27.

Red Friesian 1963.

Sodium.

The animal gained sodium at an average daily rate of 1•2 grams over the first 40 days, but with an apparent decrease in the rate; then losses occurred when the platelets were at their lowest and the leucocytes were falling between days 40 and 60 (19/6/63-9/7/63). Possibly the large deficit between days 50-60 (29/6/63-9/7/63) is due partly to the fall in intake. Facilities were not available for weighing a calf at this stage but the animal would be approximately 150 Kg. i.e. 132 fat free weight so that assuming a sodium content of 65m.eq./Kg. (Comar and Bronner 1964) the total body sodium would be of the order of 200 grams. Total loss between days 40 and 60 was 31•5 grams representing an approximately 15% loss.

The loss occurred through the urine since no rise in faecal output can be seen on the graph, the faecal output closely follows the intake; but a rise in urine level can be seen clearly.

Potassium.

Potassium loss started with the platelet fall on day 20 (30/5/63) being heaviest during the period day 50-60 (19/6/63-9/7/63) when the platelets were lowest and the leucocytes were falling, however the intake fell too during this period and the deficit could indicate a normal delay in adjustment. The loss occurred through the urine, the faecal output being related to the intake. The urine output of Potassium decreased during the rise in blood cells after day sixty, but the total potassium loss during the period day twenty to day sixty (30/5/63-9/7/63) was 102 grams, which would represent a loss of 20-25% of the animal's total

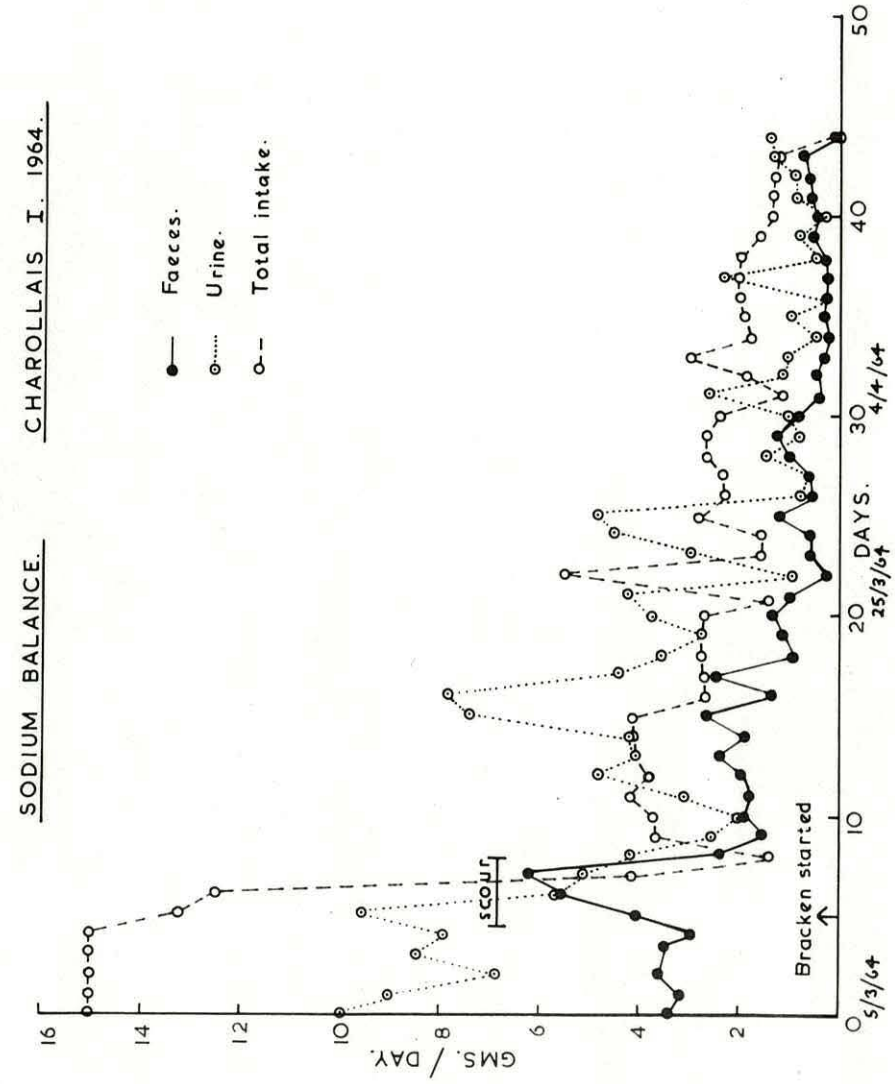

SODIUM BALANCE.
CHAROLLAIS I. 1964.
Faeces.
Urine.
Total intake.
GMS. / DAY.
16
14
12
10
8
6
4
2
0
scour
Bracken started
5/3/64
10
20
25/3/64
30
4/4/64
40
50
DAYS.

Fig. 28.

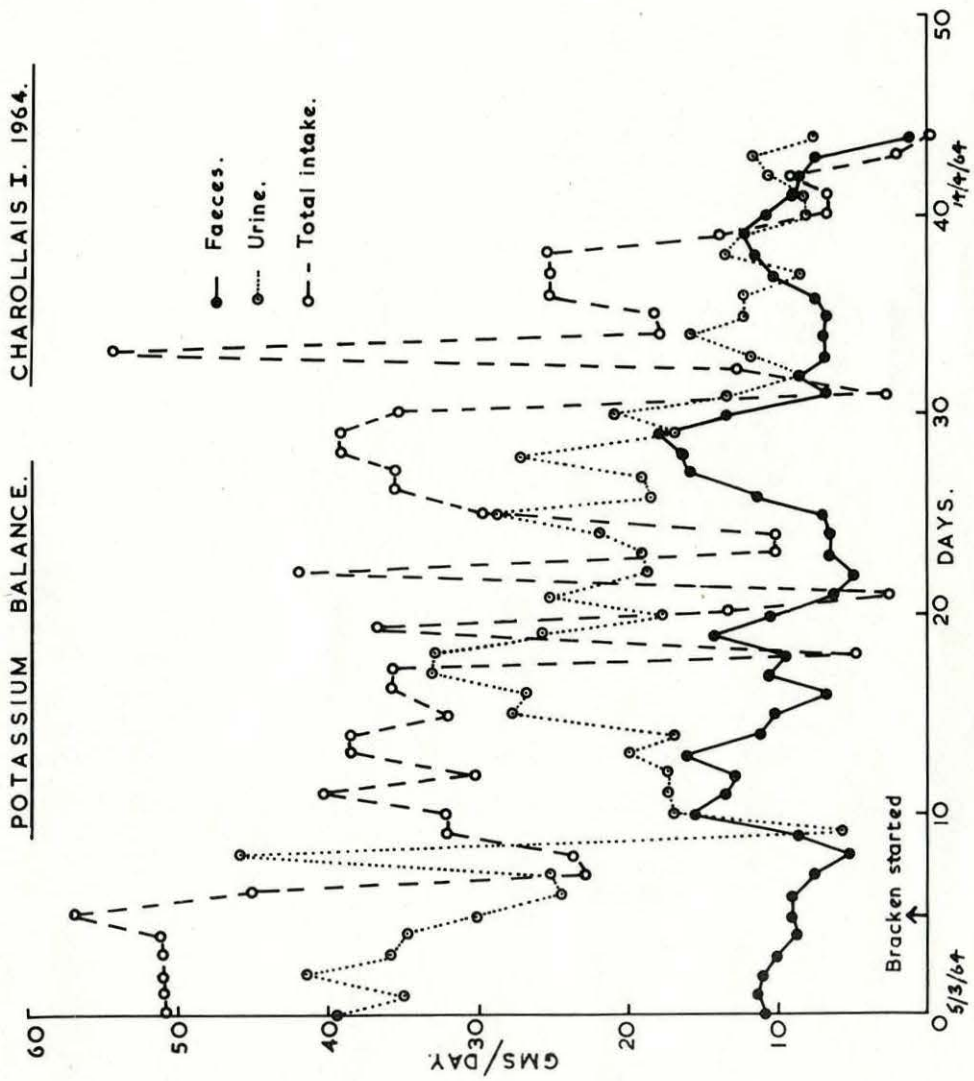

POTASSIUM BALANCE.
CHAROLLAIS I. 1964.
Faeces.
Urine.
Total intake.
GMS/DAY
DAYS.
Bracken started
5/3/64
14/4/64
0
10
20
30
40
50
60

Fig. 29.

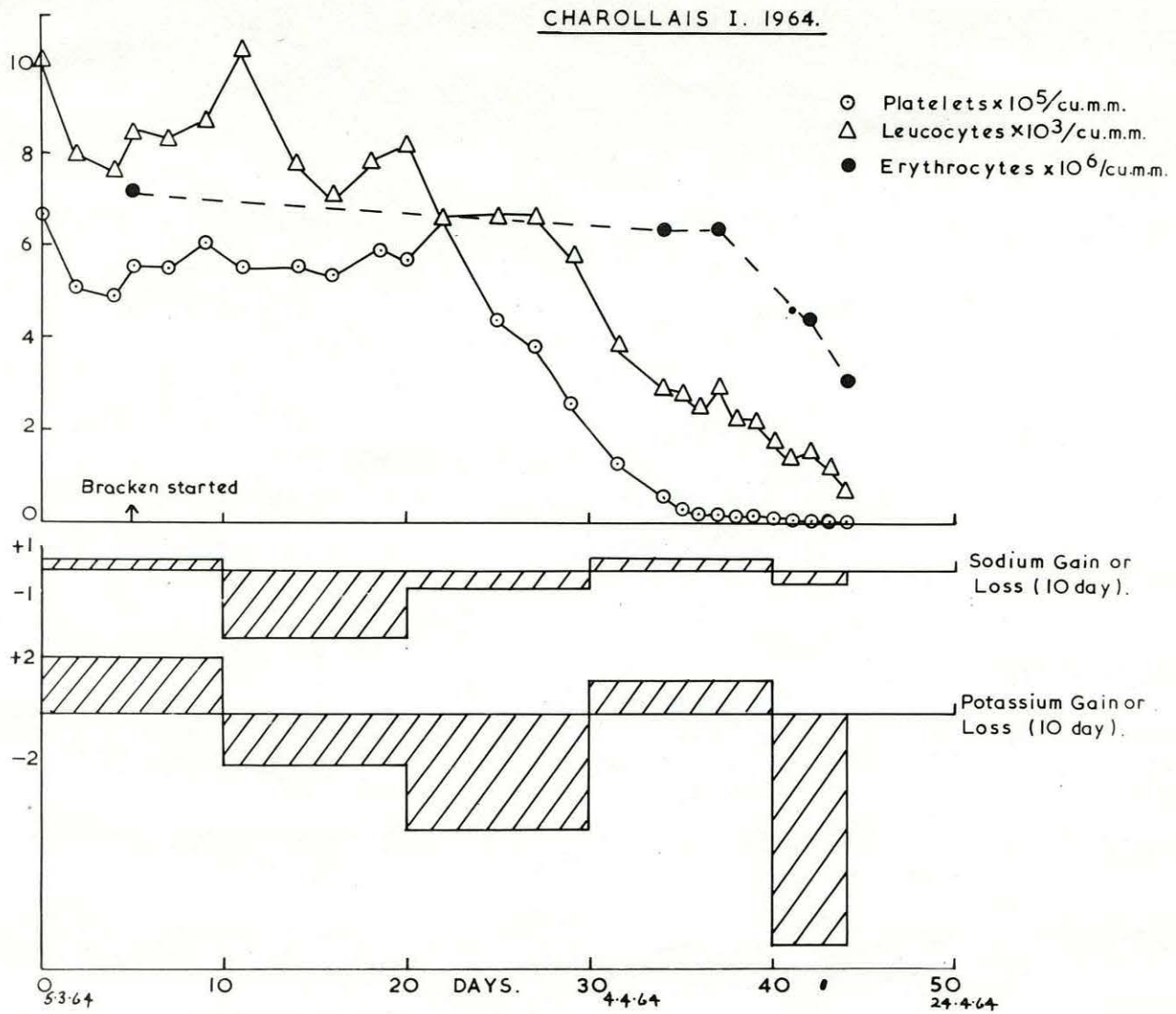

CHAROLLAIS I. 1964.
Platelets x 10^5/cu.m.m.
Leucocytes x 10^3/cu.m.m.
Erythrocytes x 10^6/cu.m.m.
Bracken started
Sodium Gain or Loss (10 day).
Potassium Gain or Loss (10 day).
DAYS.
5.3.64
4.4.64
24.4.64

Fig. 30.

potassium, assuming a content of 75 m.eq./Kg. fat free tissue (Comar and Bronner 1964).

Charollais I.

Sodium.

The animal showed a large loss of sodium (36•5 grams ) between the days ten and thirty and a further loss terminally. The figure of 36•5 gms, would correspond to a 20% loss in the 135 Kg. calf. The loss was by way of the urine which shows a clear increase at this period. The faecal output shows a gradual fall parallel to the intake.

The period during which the animal scoured was quite clearly marked by the rise in faecal sodium excretion.

Potassium.

The animal showed a very heavy potassium loss over the same period as the sodium loss, again through the urine, the total loss of the period 10-30 days (15/3/64- 4/4/64) was 71•5 grams, 20-25% of the animal's total potassium. The major loss occurred during the period of platelet fall and there was a definite rise in urine output. The faecal content was related very closely to the intake, with no increase during the period of diarrhoea analogous to the sodium loss. A large loss (57•6 gms.) in potassium occurred during the terminal period.

Friesian 1964.

This calf was unusual in that no significant salt loss occurred except during the terminal period. The animal was suffering from the effects of a C.Renale infectio throughout the experiment, and although antibiotic therapy was given the urine was found to contain a considerable amount of albumin. The heavy potassium gain compared with the slight overall sodium loss, could be explained by a loss of kidney function, leading to the failure of the preferential excretion of potassium which allows the conservation of sodium.

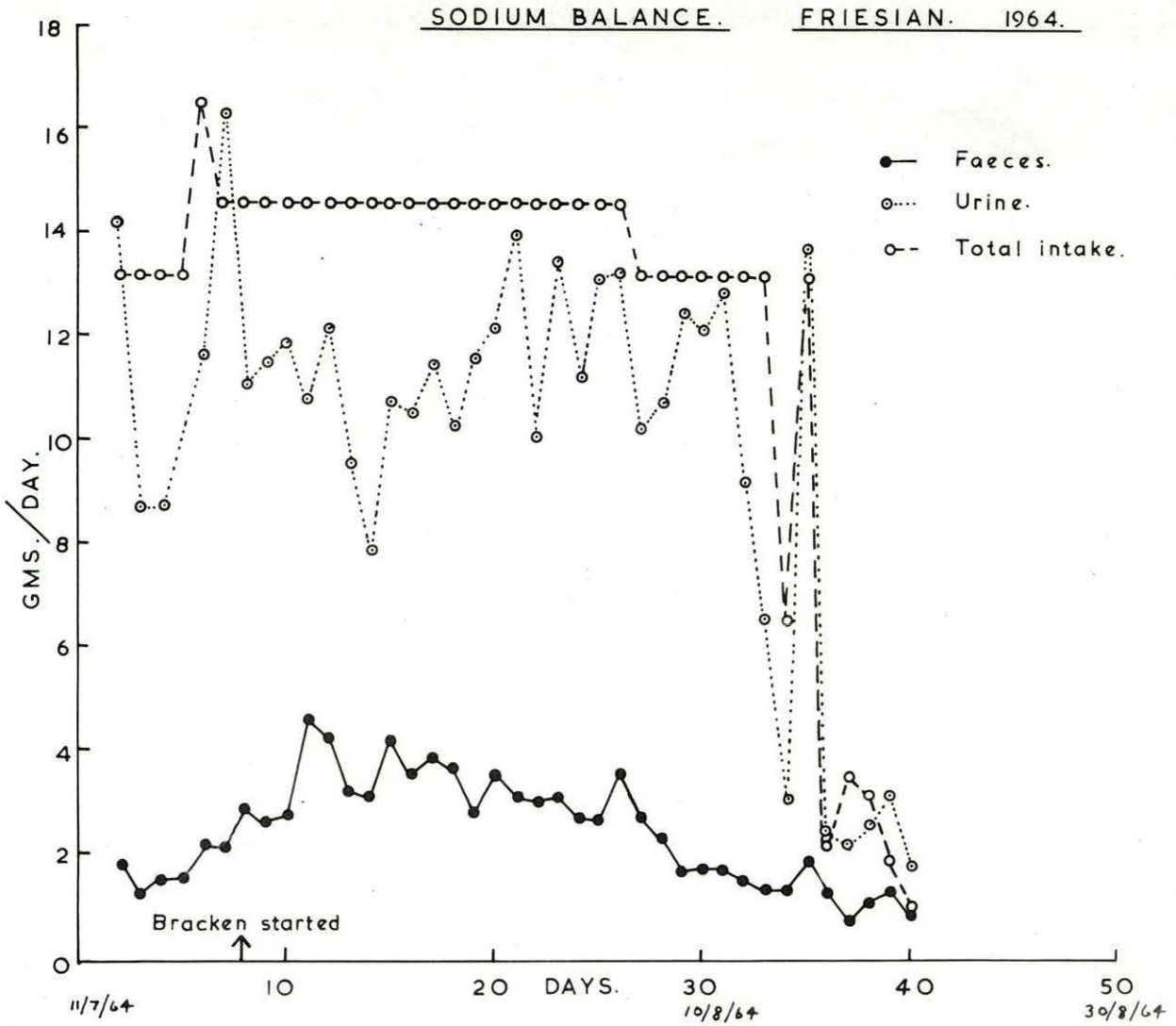

SODIUM BALANCE. FRIESIAN. 1964.
Faeces.
Urine.
Total intake.
GMS./DAY.
18
16
14
12
10
8
6
4
2
0
Bracken started
10
20
DAYS.
30
40
50
11/7/64
10/8/64
30/8/64

Fig. 31.

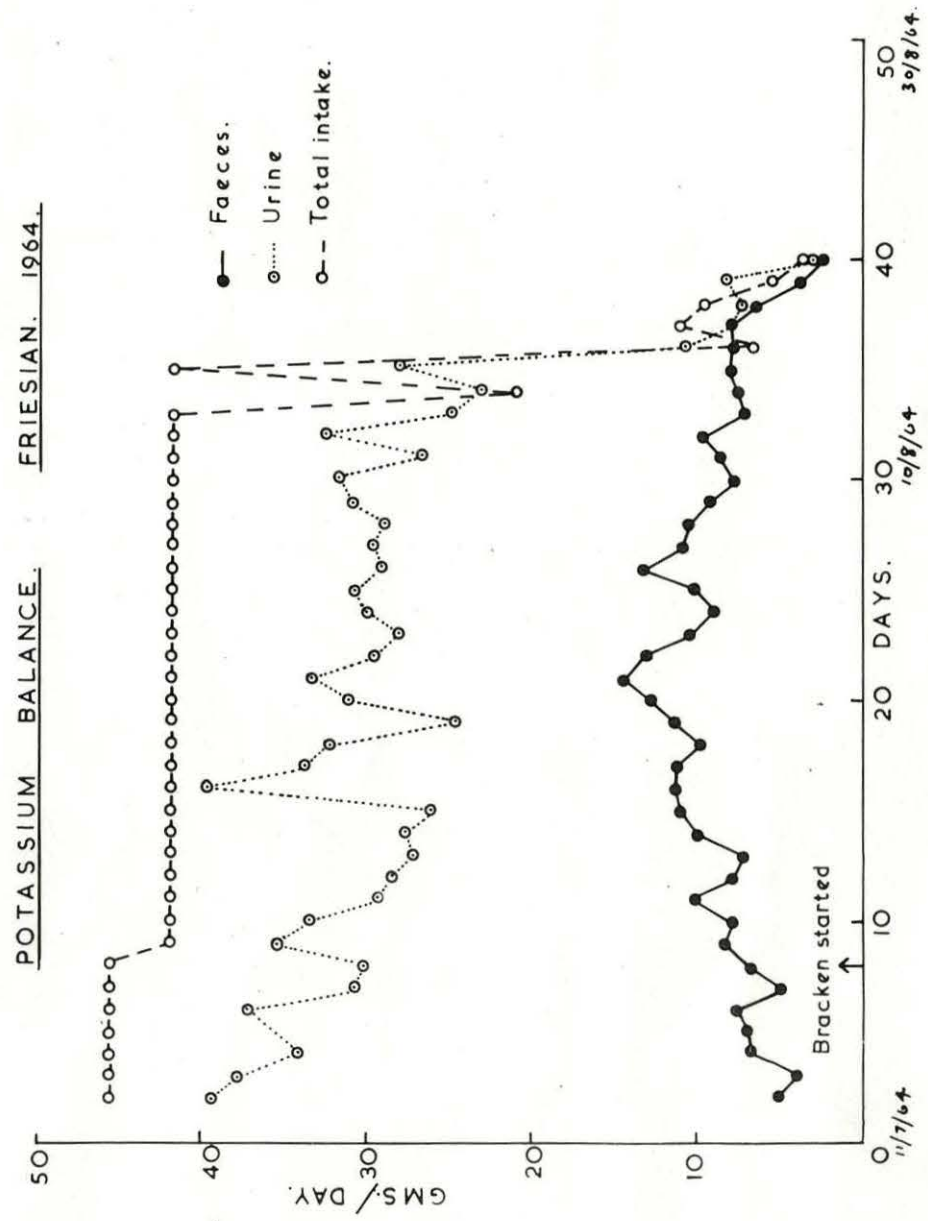

POTASSIUM BALANCE. FRIESIAN. 1964.
Faeces.
Urine
Total intake.
GMS./DAY.
50
40
30
20
10
0
11/7/64
10
20
DAYS.
30
10/8/64
40
50
30/8/64
Bracken started

Fig. 32.

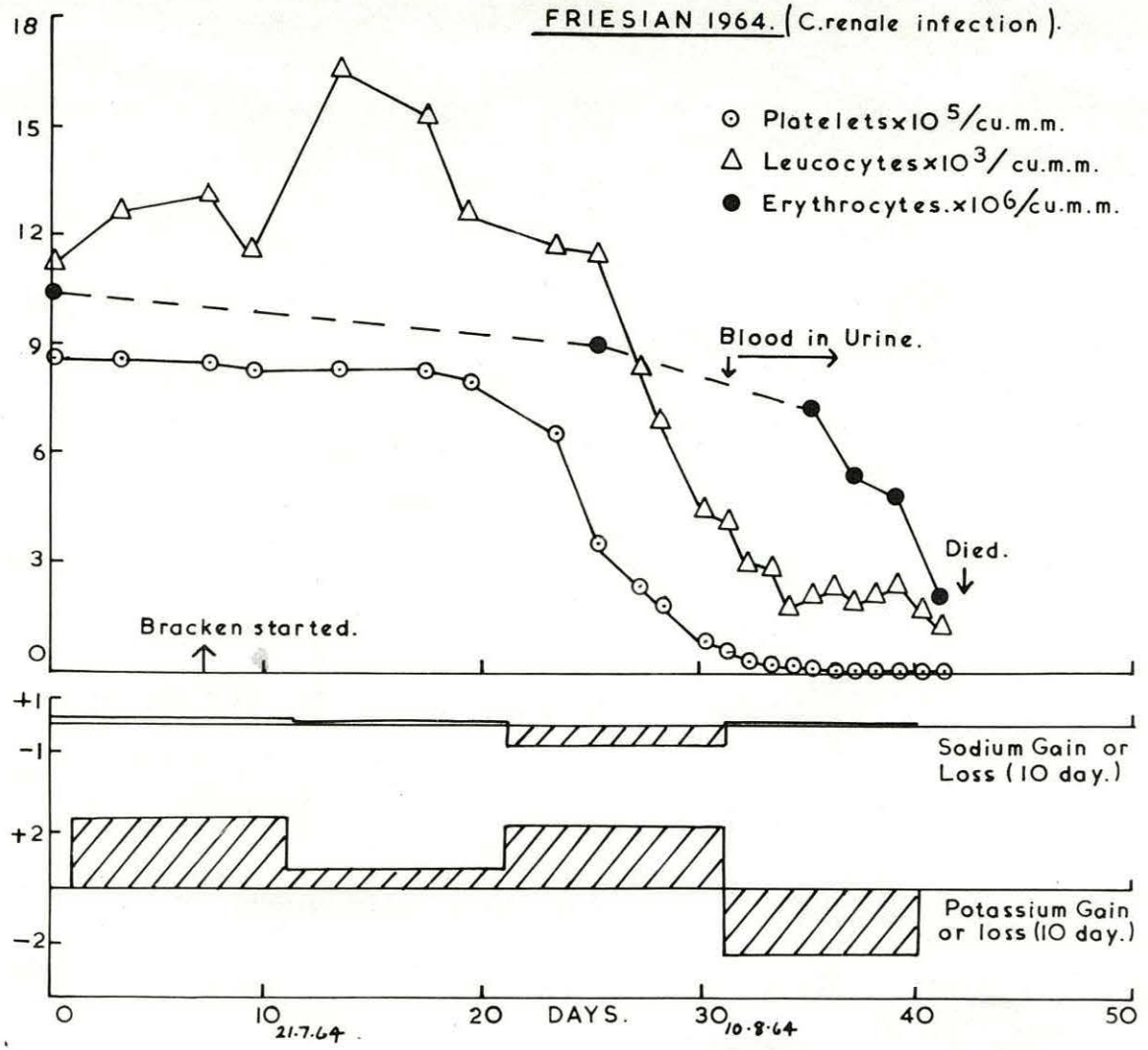
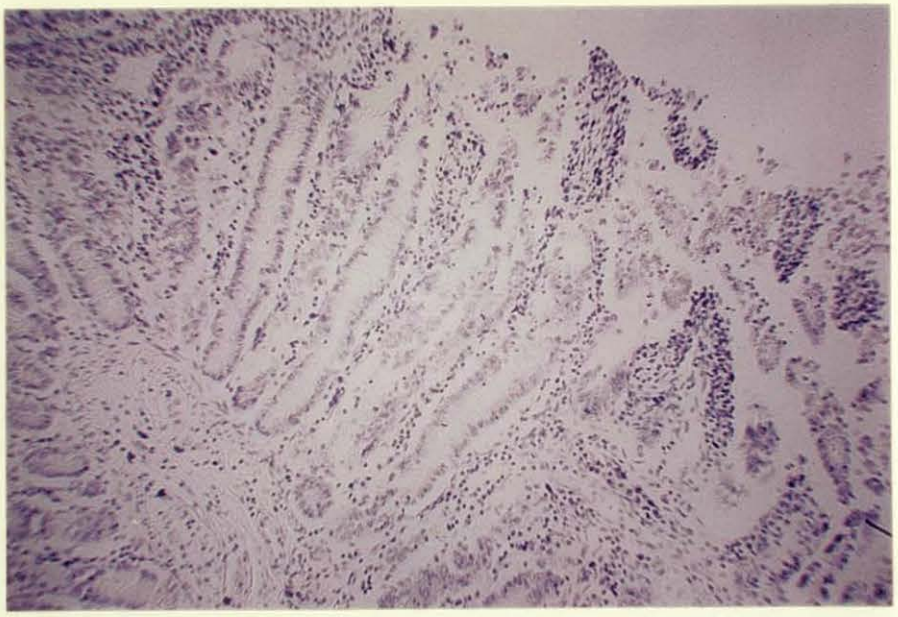
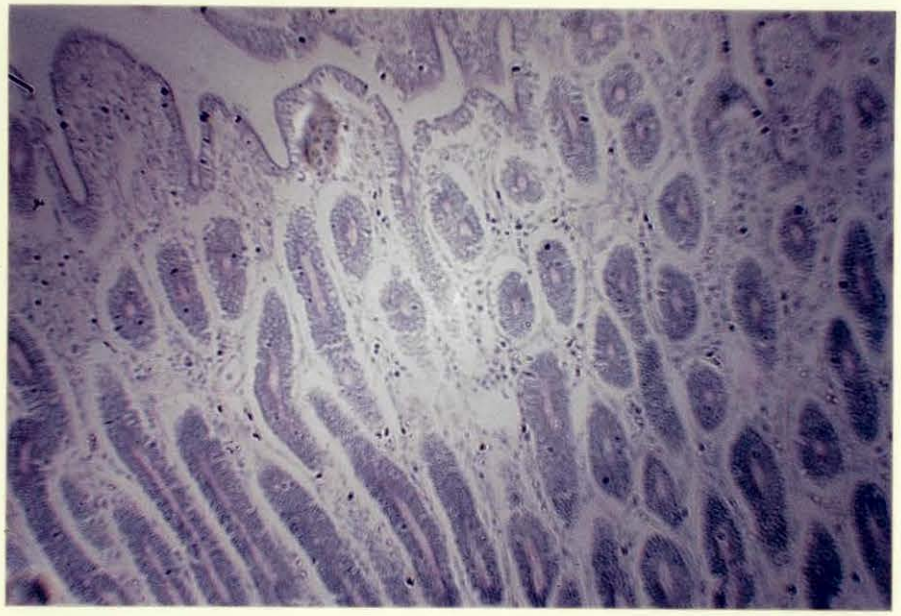

FRIESIAN 1964. (C.renale infection).
Plateletsx10 5/cu.m.m.
Leucocytesx10 3/cu.m.m.
Erythrocytes.x10 6/cu.m.m.
Blood in Urine.
Died.
Bracken started.
18
15
12
9
6
3
0
+1
-1
+2
-2
Sodium Gain or Loss (10 day.)
Potassium Gain or loss (10 day.)
0
10
21.7.64
20
DAYS.
30
10.8.64
40
50

Fig. 33.



Small Intestine.
(Charollais I), 7μ section, x 100, Wright's stain.



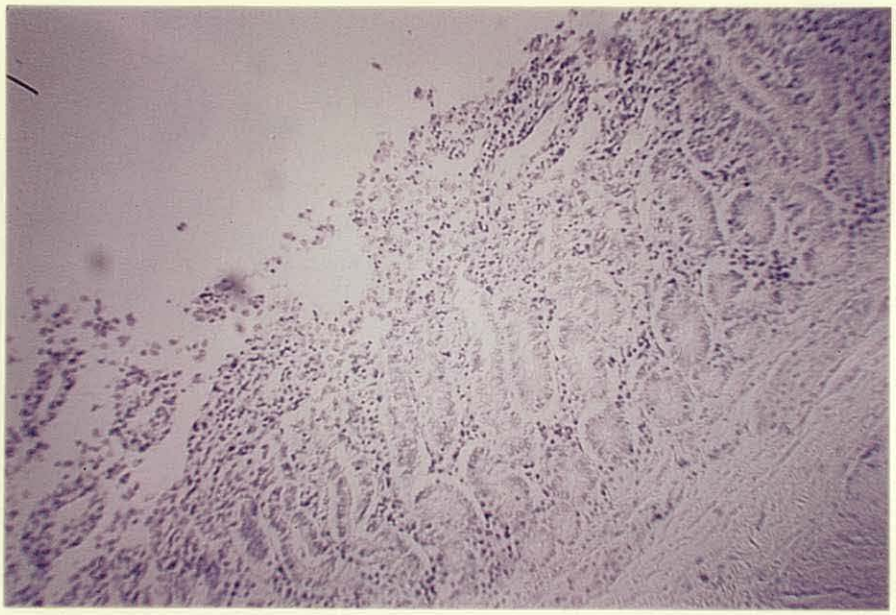
Small Intestine.
(Normal Calf one month old), 7μ section, x 100, Wright's stain.

Histological evidence.

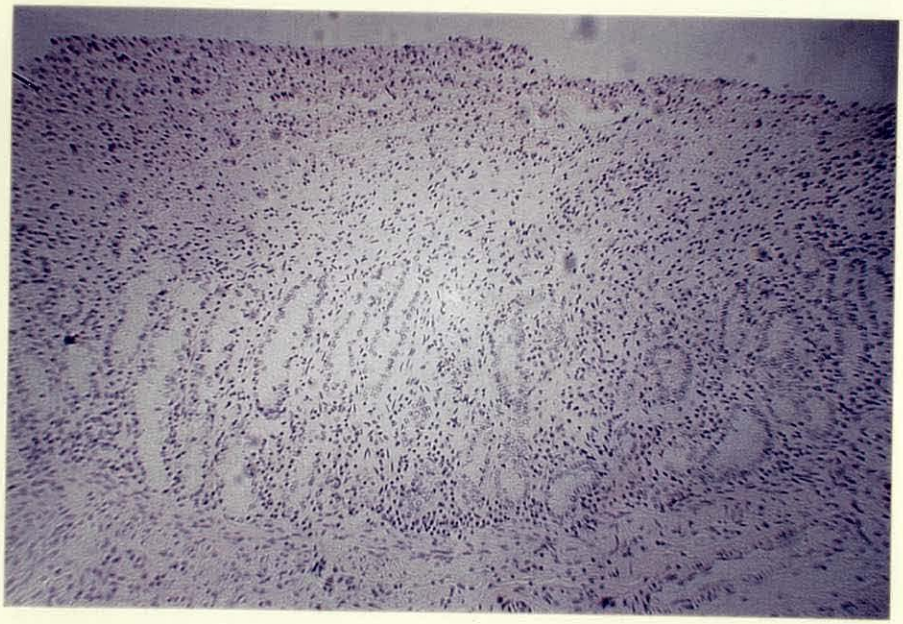
Histological examination of sections of small and large intestine was made on the Charollais I and the Friesian Calf just described, as well as the Calf 152 discussed in the previous section.

All three animals showed the same type of injury, similar to the changes seen after irradiation. The nature of these changes, as indicated by the plates, was characterized by an unmistakable loss of activity and structural cohesion of the epithelial cells. The villi were very short and in fact the gross structure was retained only at the bottoms of the crypts near the muscularis mucosa. The tips of the villi were structureless and simply an unorganized confusion of cells, among which it was possible to recognise the epithelial cells although they were no longer forming a continuous layer. Mitoses were extremely rare, even at the base of the crypts and the normal goblet cells were absent. Plasma and lymphocytic type cells were a prominent feature.

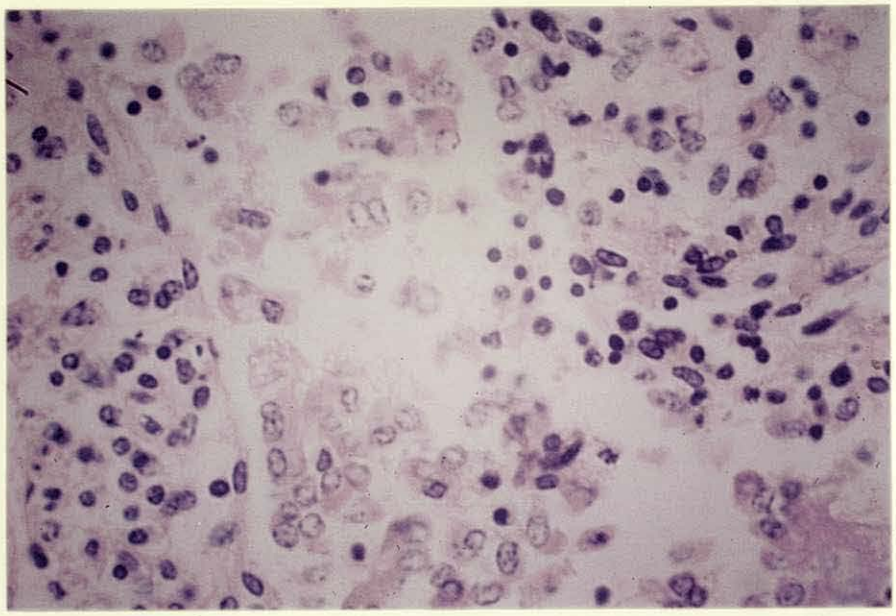
Compared with sections from a normal calf, the villi were about $\frac{1}{4}$ of the normal length and the epithelial cells far more shrunken and much less basophilic. In the normal calf preparations all the usual features could be seen, including frequent mitosis, active goblet cells etc. Bacteria were not observed in any of the sections examined.



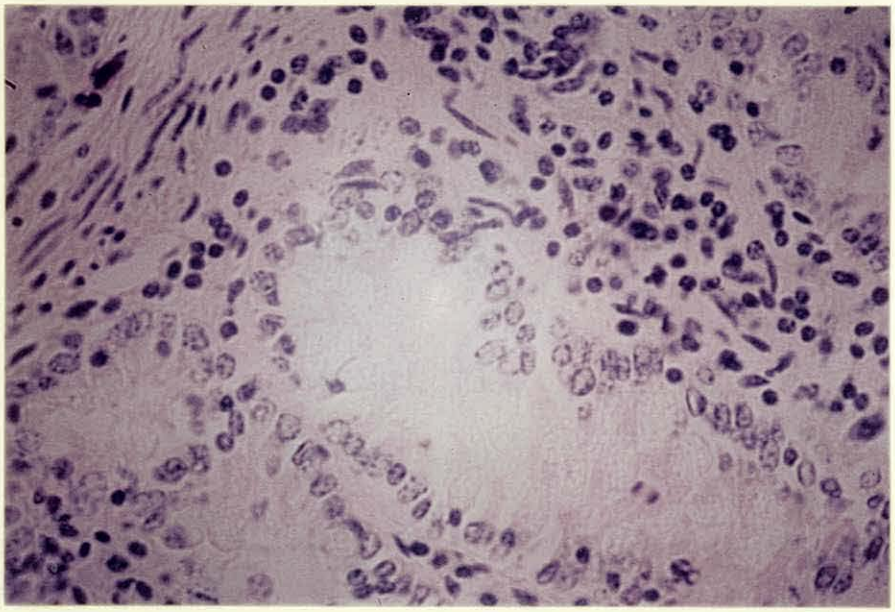
Duodenum.
(Charollais I). 7μ section, x 100, Wright's stain.



Small Intestine.
(Friesian 1964), 7μ section, x 100, Wright's stain.



Small Intestine. Sloughing of epithelial cells.
(Calf 152). 7μ section, x 400, Wright's stain.



Small Intestine. Crypt Mucosa.
(Friesian 1964). 7μ section, x 400, Wright's stain.

Discussion.

The figures obtained for the sodium and potassium balance were considered as functions of ten day periods since it was felt that this would eliminate the normal daily fluctuations, however, the general trends and the specific daily values can be seen in the graphs, the tables in the appendix record the daily figures. Workers with children have found 25% variation from the mean rate of gain; periods of retention oscillating with periods of loss. (Black and Thompson 1951), so that in order to estimate the true rate of gain the experimental observation should be over long periods.

The results indicate that there is no serious loss of either sodium or potassium through the intestine, in all the three animals studied faecal output was closely related to the intake. Where an animal did develop diarrhoea (Charollais I) the loss was clearly visible. The cause of the animal's scouring was possibly related to the sensitivity state of the calf to bracken or more probably due to an intestinal infection or the change of diet, since the Calf 152 (Section I) presensitized in a similar way did not scour.

The losses which did occur were through the urine and the potassium loss can be related to cell breakdown, the Red Friesian and the Charollais I which suffered the large urine losses showed the pronounced tissue manifestations such as mast cell increases and petechial haemorrhages (Section I). The Friesian (1964) which did not exhibit salt losses was unique in the animals studied in that hardly any tissue reactions were observed, however, the failure of kidney function cannot be discounted.

It is not clear from the experiments if the apparent negative balance observed in all the animals terminally was due to the fall in appetite and consequent decrease in intake, or if in fact the fall in appetite was caused by the changes which brought about the loss.

Although the sodium and potassium balances did not reveal any marked intestinal loss, it is evident from the histological examinations of post mortem samples that extensive degeneration of the intestinal tract did occur. Although the amount of bracken given was probably too small to produce the 5 day gastro-intestinal syndrome, it was possible that since the dosage was continuous, a cumulative effect could have resulted in a similar acute syndrome. Possibly the development of diarrhoea is necessary before serious loss occurs and this did not appear in any of the animals. Crouch and Overman (1961) found that the persistent diarrhoea may have affected the survival of irradiated monkeys, although this was not the immediate acute type and appeared when the bone marrow was apparently recovering.

Although extensive salt loss by way of the intestinal tract did not occur the damage and loss of surface area was such that the absorptive function must have been impaired, The lesions were very similar to those observed after irradiation and similar gross disfunctions such as intussusceptions and tenesmus were observed. The organs were also distended and filled with fluid in the same manner.

Naftalin and Cushnie (1954), the only other workers to describe the gross pathology of the intestinal tract, observed extensive bacterial invasion throughout. This was not seen in the animals described in this thesis, probably because all the animals were treated with antibiotic

although occasional ulcers were observed.

In summary, the damage to the intestinal tract must play an important part in the bracken poisoning syndrome, especially in cases where antibiotic therapy is not given. This would seem essential in view of the thinness of the epithelial layer. Other therapeutic measures may be beneficial especially if diarrhoea developed in an animal, some workers do report diarrhoea although this does not seem to be a regular feature, in these cases parenteral saline and possibly aldosterone therapy would be beneficial.

The importance and degree of loss of function is difficult to assess, the animals do not appear to suffer a marked loss of weight during the syndrome and it is probable that provided other factors are favourable the animal could exist on its reserves until regeneration of the intestine took place.

# SECTION III.

## DELAYED EFFECTS.

## Introduction.

Many radio-mimetic chemicals are known to have effects analogous to the long term effects of irradiation. Alkylating agents are known to produce gene mutations, and in many cases the genetic effects produced by irradiations and chemical mutagens are very close (Bacq and Alexander 1961). Similarly, strains of E. coli resulting from survivors of heavy doses of X rays, are resistant to nitrogen mustards as well as to further doses of irradiation, this production of cross-resistance could indicate a close similarity between the two toxic agents.

Radiation carcinogenesis can follow whole body exposure but the site and incidence of tumours is highly dependent on the genetic make up of the animals and varies widely between strains. The abdominal region of the rat appears to be sensitive to tumour induction after large doses of irradiation (Bacq and Alexander 1961, and Maisin et al. 1957).

In view of the immediate effects of bracken ingestion, it was thought possible that the plant could have the ability to produce more delayed effects, if fed directly. Therefore at the beginning of this work, two female Wistar rats (weighing 100 grams each) were fed with milled rat cake (60%) and milled bracken rhizome (40%); the rhizome powder was later shown to have only low activity with respect to the bovine haemopoietic syndrome. The rats were fed this diet from 3/4/63 - 7/6/63 with $B_1$ therapy, without any apparent effects, but 12 months after the commencement of the diet it was noted that the animals had considerable vaginal haemorrhages, which continued intermittently for the following months. The animals were killed

in June 1964 and Nov. 1964 respectively and were found to have haemorrhagic nodules on one of the uterine horns.

It was thought worthwhile to test the activity directly on a much larger number of rats, using bracken of known activity with respect to the bovine haemopoietic syndrome.

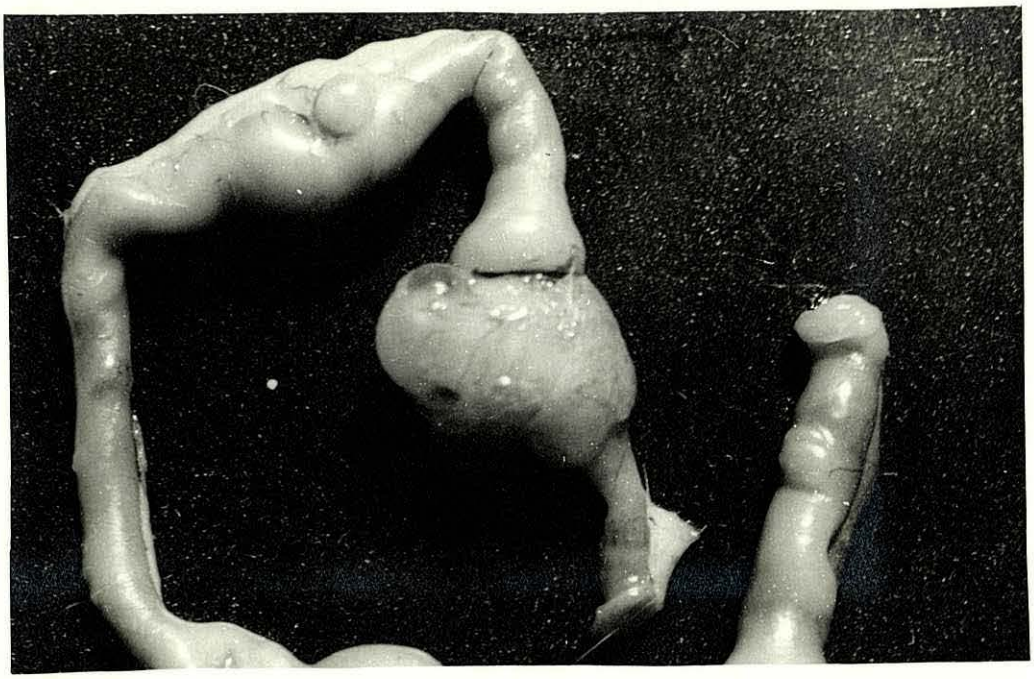
EXPERIMENTAL.

The June 1964 milled powder described in the previous section was made into pellets with Levers No.4 rat diet (66% No.4, 33% bracken powder by weight). This diet was fed seven-week old, hooded, Lister non-inbred rats purchased from Glaxo Ltd., twenty of each sex were used (male weight 125 grams, female 110 grams). It was found impossible to estimate the amount consumed due to wastage incurred, but since no other food was offered and the diet fed ad. lib. this would approximate to the animals normal dietary requirements.

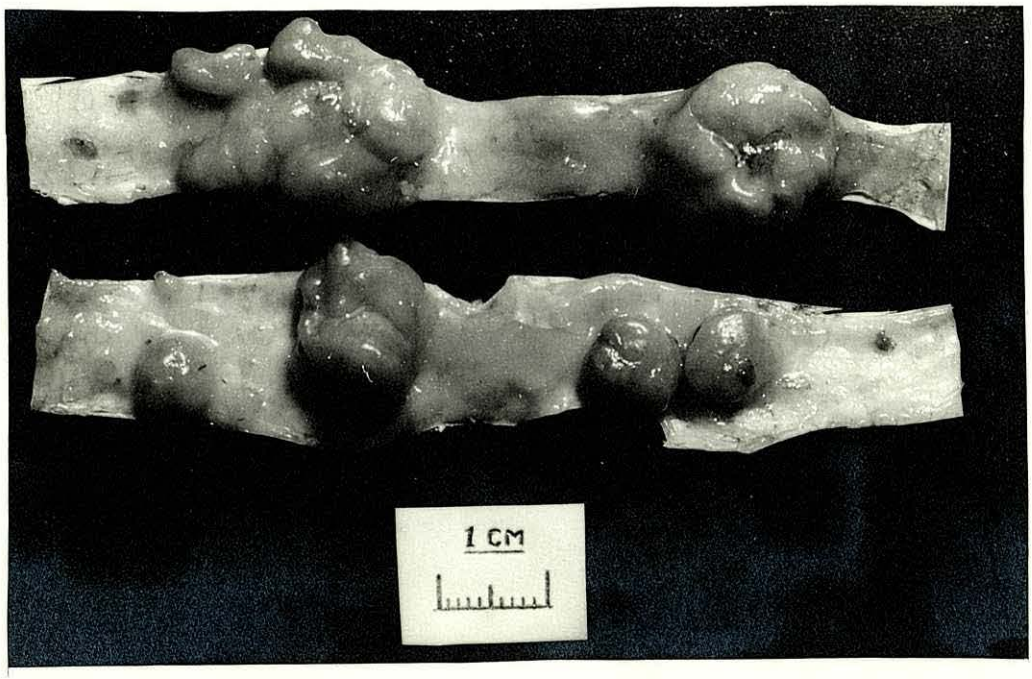
The diet was made up in small batches and dried immediately to avoid mould growth.

The diet was fed 64 days from the 14/9/64 to the 17/11/64 inclusively, and the 8-18/1/65. Since bracken also has a thiaminase enzyme quite distinct from the "cattle factor" (Evans W.C., et al. 1958) it was necessary to provide extra-dietary $B_1$. This was given by subcutaneous injection of 3 mgm. of $B_1$ in 0·2 mls. of physiological saline / rat on the 30/9/64, 14/10/64 and the 30/10/64 respectively while the animals were under light ether anaesthesia. 40 control rats of the same age and weight receiving the normal rat diet were given the same treatment.

White rats were also used, two as controls, four receiving the diet.



Typical external appearance of intestinal tumours. (Ileum).




Same length of intestine opened out.

Fig. 34.

80 Swiss non-inbred white mice, equal numbers of each sex, were fed the bracken diet and normal diet alternately because of the labour involved in giving both the 80 experimental and 70 controls $B_1$ injections. Bracken was fed 7-15/9/64, 21-27/9/64, 2-9/10/64, 16-23/10/64, 7-18/1/65 and 26-29/1/65.

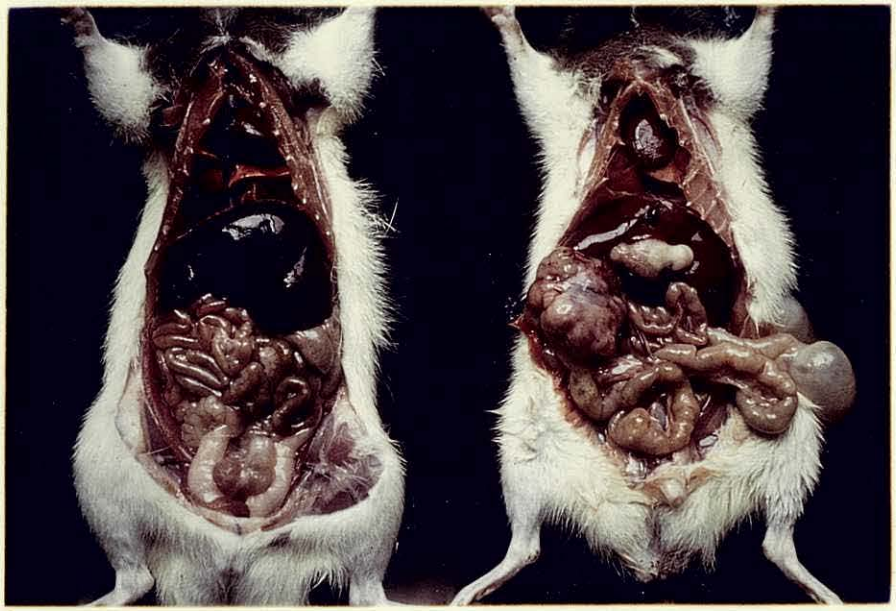
RESULTS.

Rats.

The first appearance of any lesions was observed 29 weeks after the start of the experiment when a male rat died after scouring, another male in poor condition was killed on the same date. Post mortem examination of these and subsequent animals has revealed the following typical lesions.

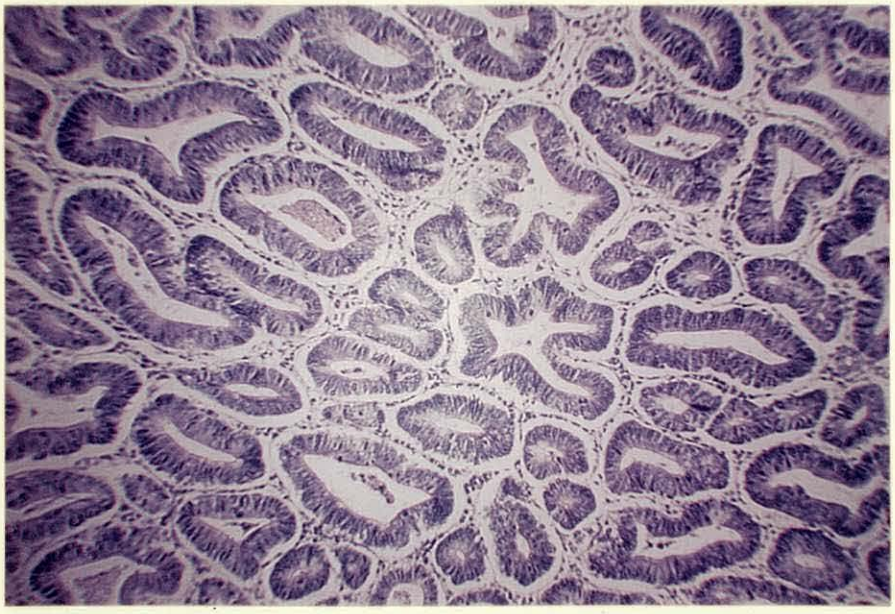
There were numerous multiple tumours protruding into the lumen of the intestine; occurring throughout the small intestine but chiefly in the ileal region. While most are of the order of 5-18 mm. in diameter, (Fig. 34), 16 of the animals had developed a very large tumour 2-4 cms. in diameter (Plate) as well as the other smaller widespread tumours. Frequently the larger tumours protruded out through the muscle wall of the intestine, often with adhesions to the surrounding tissues and organs. Some of the animals scoured badly and were usually found to have intussusceptions caused by one of the nodules; in other cases a diverticulum or a ballooning of the intestine has been found.

The only animal not conforming to the general pattern was a female which developed a mammary tumour.



Control Rat.

Rat fed Bracken.
(Large tumour).



Nodule. Ileum.
Rat Male. 22/1/65. 7μ section. x 100, Wright's Stain.

The tables in the appendix illustrate the results and it can be seen that all the experimental animals have died or have been killed in poor condition. In every one of the rats lesions of the type previously described were found. No control animals have shown any effects, while all of the 4 white rats fed bracken have developed similar lesions.

Histological examination of these tumours has revealed proliferation of the epithelial layer of the intestine, numerous mitoses can be seen in the columnar cells (Plate ) and while the original structure of the villi was visible the cells were more closely packed and several layers thick.(Plate ). In the smaller nodules the central connective tissue seems to increase with the villi, but in the larger tumours little connective tissue can be seen.

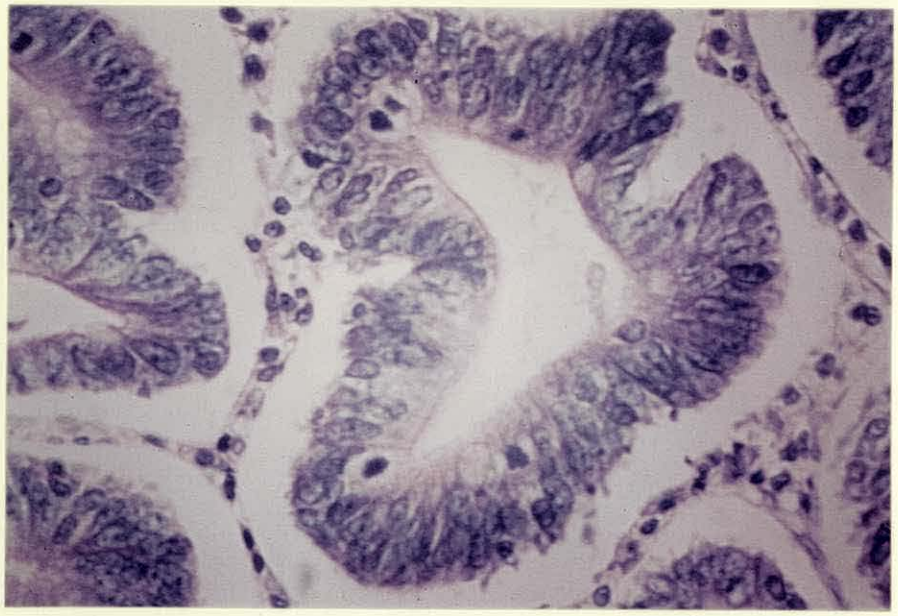
Dr.R.Schoental of the Toxicology Research Unit, Carsharlton, has kindly examined both animals and sections prepared from the tumours and confirmed the presence of malignant carcinoma of the intestinal mucosa.

Mice.

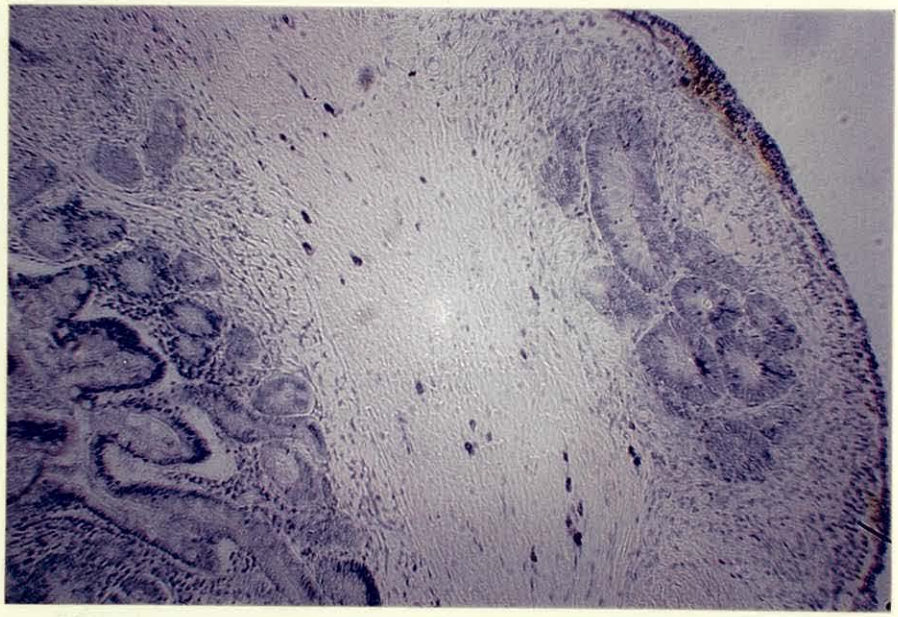
No lesions have been observed in the bracken fed mice although many male mice of both bracken and control groups have died apparently as a result of urethral blockage by bladder calculi.

Discussion.

The evidence presented indicates that the bracken content of the diet has induced tumours in rats. The mechanism by which the tumours are induced will require further investigation since there were at least two possible



Nodule. Ileum. Showing mitoses.
Rat Male. 22/1/65. 7μ section. x 400, Wright's stain.



Small Intestine. Metastased Tumour.
Rat Male. 22/1/65. 7μ section. x 100, Wright's stain.

complications, mechanical damage of the intestine by the bracken, and the effect of the thiaminase enzyme. The first although not ruled out is unlikely since the bracken was fed as a milled powder and only the soft fleshy leaf material was used. The effect of the thiaminase enzyme is unlikely since the animals were given $B_1$ therapy and no clinical $B_1$ deficiency developed, no record can be found of any relationship between sub-clinical or acute $B_1$ deficiency and tumour induction.

The feeding of a protein-free soluble extract would have eliminated these factors, but at the time of the experiment no chemical procedure was known which would consistently give a high yield of the cattle haemopoietic factor. The possible effect of the $B_1$ injections and anaesthesia was not considered since none of the control animals have developed any lesions.

Only a few of the females given June 1964 bracken have developed lesions of the uterine horns, but it should be noted that these lesions took longer to develop than the tumours i.e. 12 months compared with 6 months. Conversely, no tumours were observed in the two original females, but it should be noted that the rhizome powder used was far less active with respect to the bovine than the June 1964 material.

It is not clear whether the particular site of the tumours is caused by a preferential absorption in that area or merely a greater sensitivity of the rat intestine to tumour induction (Schoental and Bensted 1963, Bacq and Alexander 1961), further work using different administrative routes should clarify this, by avoiding primary contact with the intestines.

Parker and McCrea (1965 personal communication) noted during the course of a survey that sheep losses in bracken areas of Yorkshire were due to three factors, Bracken poisoning, parasite infestations and adenocarcinoma of the small intestine. However, Rosenberger in 1960 reported the presence of haematuria in cattle fed bracken hay over long periods and that changes of a polypous-tumorous nature occurred in the bladder mucosa, while Giorgiev (1963) obtaine an extract from the urine of cattle fed hay from haematuria districts, which, when introduced into the bladders of dogs produced changes similar to haemangioma. The same extract when applied to the skin of white mice produced excrescences similar to papilloma.

The possible common identity of the cattle haemopoietic factor and the factor causing the rat tumours will require investigation by feeding of purified extracts of the plant.

The possibility of a relationship between an antigen-producing agent and a carcinogen is viewed with great interest in the light of the concept proposed by Green (1954) and the work summarized by Alexander (1965). Green suggested that chemical carcinogenesis is the result of an antibody induced by a carcinogen-protein complex deleting marker sites on cell surfaces. Anderson (1964) found that chemical carcinogenesis could be altered by varying the psychological states of experimental rats injected with 20-methyl-cholanthrene, she concludes that the reduced tumour induction in stressed rats was due to a diminished immunological response.

Fjelde and Turk (1965) showed an immunological response was evoked in local lymph nodes by chemical carcinogens, and that the development of contact sensitivity

paralleled the susceptibility to skin carcinogenesis; this supplements the work of Anderson (1963) who showed that temporary inhibition of the immunological response of the draining lymph nodes by irradiation and other agents delayed carcinogenesis. It is possible that the ionizing radiations and radio-mimetic chemicals all produce tumours by provoking immunological reactions, the long delays before the appearance of the lesions seen in some cases could be due to a decrease in tolerance with age, in the manner suggested by Hotchin and Collins (1964).

It is felt that this aspect is worthy of further investigation.

# APPENDIX

MALE RATS.

| Date. | Scouring | Intuss-usception | Multiple tumours | Large tumour | Other features |
|---|---|---|---|---|---|
| 22/4/65 Died | + | - | + | - | |
| 22/4/65 Killed | + | + | + | - | |
| 29/4/65 Killed | + | - | + | - | |
| 8/5/65 Killed | + | - | + | - | |
| 21/5/65 Died | + | + | - | - | |
| 22/5/65 Killed | + | + | + | - | Caecum enlarged. |
| 24/5/65 Died | - | - | + | + | Perforation of intestine |
| 2/6/65 Killed | + | - | + | - | |
| 8/6/65 Killed | + | - | + | + | |
| 8/6/65 Killed | - | - | + | - | |
| 8/6/65 Killed | - | - | + | - | |
| 10/6/65 Killed | - | - | - | + | Diverticulum |
| 10/6/65 Killed | - | - | + | + | |
| 10/6/65 Killed | - | - | + | - | |
| 21/6/65 Killed | - | - | + | - | |
| 30/6/65 Killed | - | - | - | + | |
| 1/7/65 Killed | - | - | + | + | |
| 15/7/65 Killed | + | - | + | + | |
| 21/7/65 Killed | + | - | + | - | |

## FEMALE RATS.

| Date | | Scouring | Intussusception | Multiple tumours | Large tumour | Haemorrhagic Uterine horns |
|---|---|---|---|---|---|---|
| 24/11/64 | Killed | - | - | middle ear infection. | - | - |
| 17/5/65 | Died | - | - | - | + | - |
| 19/5/65 | Died | + | + | 1 small nodule | - | - |
| 29/5/65 | Killed | + | - | + | + | - |
| 10/6/65 | Killed | - | - | - | Mammary | - |
| 21/6/65 | Killed | + | - | + | + | - |
| 25/6/65 | Killed | + | + | + | - | - |
| 30/6/65 | Killed | - | - | + | 2 | - |
| 1/7/65 | Killed | - | - | + | + | ? |
| 1/7/65 | Killed | - | - | + | - | ? |
| 1/7/65 | Killed | - | - | - | + | - |
| 1/7/65 | Killed | - | - | + | - | + |
| 13/7/65 | Killed | - | - | - | + | - |
| 15/7/65 | Killed | + | - | 1 + detached in caecum | - | ? |
| 21/7/65 | Killed | + | - | + | - | ? |
| 28/8/65 | Killed | - | + | + | - | - |
| 28/8/65 | Killed | - | - | + | + | - |
| 28/8/65 | Killed | - | - | + | - | - |
| 28/8/65 | Killed | - | - | + | + | - |
| 28/8/65 | Killed | - | + | + | - | - |

Red Friesian 1963. Balance Experiment.

| | Sodium (gms./day). | | | | Potassium (gms./day). | | | |
|---|---|---|---|---|---|---|---|---|
| Date. | Faeces | Urine | Intake | ± | Faeces | Urine | Intake | ± |
| 10/5/63 | 6•0 | 7•65 | 12•8 | - •85 | 8•12 | 23•4 | 31 | - •52 |
| 11/5/63 | 5•7 | 6•16 | 13•1 | + 1•14 | 7•24 | 18 | 27•75 | + 2•51 |
| 12/5/63 | 5•6 | 4•62 | 12 | + 1•8 | 9•9 | 18•05 | 31•75 | + 3•75 |
| 13/5/63 | 6•17 | 6•00 | 12 | - •17 | 12•0 | 18•7 | 35•24 | + 4•54 |
| 14/5/63 | 5•3 | 4•8 | 12 | + 1•9 | 12•35 | 18•8 | 35•24 | + 4•1 |
| 15/5/63 | 5•08 | 4•96 | 12 | + 2•0 | 11•70 | 24•2 | 36•5 | + •6 |
| 16/5/63 | 5•00 | 7•50 | 13•5 | + 1 | 10•15 | 25•6 | 36•5 | + •75 |
| 17/5/63 | 5•66 | 7•8 | 13•5 | + •04 | 9•4 | 28•1 | 36•5 | - 1•0 |
| 18/5/63 | 4•64 | 7•85 | 12•8 | + 0•3 | 8•65 | 28•2 | 36•5 | - •35 |
| 19/5/63 | 4•16 | 5•06 | 13 | + 3•78 | 9•34 | 34•0 | 36•5 | - 6•84 |
| 10 day Total + or - | | | | +11•64 | Total + or - | | | + 7•54 |
| 20/5/63 | 3•52 | 7•24 | 14•7 | + 3•94 | 8•62 | 23•5 | 38•5 | + 6•38 |
| 21/5/63 | 4•07 | 5•82 | 17•6 | + 7•7 | 8•23 | 26•25 | 43•5 | + 9•0 |
| 22/5/63 | 4•75 | 10•3 | 16•4 | + 1•35 | 10•75 | 30•6 | 39•4 | - 1•95 |
| 23/5/63 | 5•55 | 10•55 | 16•4 | + •3 | 10•93 | 26•2 | 39•4 | + 2•27 |
| 24/5/63 | 4•83 | 9•4 | 16•4 | + 2•2 | 9•4 | 26•4 | 39•4 | + 3•6 |
| 25/5/63 | 5•15 | 6•86 | 16•4 | + 4•4 | 12•05 | 20•1 | 39•4 | + 7•25 |
| 26/5/63 | 6•56 | 9•95 | 16•4 | - •1 | 15•8 | 24•8 | 39•4 | - 1•2 |
| 27/5/63 | 4•52 | 9•6 | 17•4 | + 3•3 | 13•3 | 22•5 | 39•4 | + 3•6 |
| 28/5/63 | 6•66 | 10•4 | 17•4 | + 0•34 | 18•2 | 23•8 | 39•4 | - 2•6 |
| 29/5/63 | 5•8 | 10•3 | 17•4 | + 1•3 | 14•1 | 23•8 | 39•4 | + 1•5 |
| 10 day Total + or - | | | | +24•83 | Total + or - | | | +27•85 |

Red Friesian 1963 Contd.

| Date. | Sodium (gms./day) Faeces | Urine | Intake | ± | Potassium (gms./day) Faeces | Urine | Intake | ± |
|---|---|---|---|---|---|---|---|---|
| 30/5/63 | 6•64 | 11•7 | 17•4 | - 1•0 | 17•4 | 21•4 | 39•4 | + •6 |
| 31/5/63 | 7•00 | 10•6 | 17•4 | - •2 | 14•5 | 27•8 | 39•4 | - 2•9 |
| 1/6/63 | 5•9 | 9•1 | 17•4 | + 2•4 | 13 | 26•6 | 39•4 | - •2 |
| 2/6/63 | 5•84 | 9•25 | 17•4 | + 2•3 | 16•6 | 26•6 | 39•4 | - 3•8 |
| 3/6/63 | 6•74 | 11•45 | 17•4 | - •8 | 18•6 | 22•9 | 39•4 | - 2•1 |
| 4/6/63 | 5•5 | 10•5 | 17•4 | + 1•4 | 15•7 | 27•8 | 39•4 | - 4•1 |
| 5/6/63 | 5•4 | 10•2 | 17•4 | + 1•8 | 16 | 22 | 39•4 | - •6 |
| 6/6/63 | 6•06 | 8•05 | 17•4 | + 3•3 | 15•9 | 27•6 | 39•4 | - 4•1 |
| 7/6/63 | 5•6 | 14•5 | 17•4 | - 3•7 | 13•8 | 24•6 | 39•4 | + 1•0 |
| 8/6/63 | 6•44 | 7•2 | 17•4 | + 3•7 | 14•6 | 29•6 | 39•4 | - 4•8 |
| 10 day Total + or - | | | | + 9•2 | Total + or - | | | -21.0 |
| 9/6/63 | 4•15 | 13•9 | 16 | - 2•05 | 14•3 | 26•2 | 39•4 | - 1•1 |
| 10/6/63 | 4•95 | 10•45 | 16 | + •6 | 16•2 | 22•3 | 39•4 | + •9 |
| 11/6/63 | 4•68 | 9•0 | 16 | + 2•3 | 14•8 | 27•2 | 39•4 | - 2•6 |
| 12/6/63 | 4•4 | 9•9 | 16 | + 1•7 | 15•3 | 23•1 | 39•4 | + 1•0 |
| 13/6/63 | 5•8 | 9•75 | 16 | + •45 | 13•3 | 30•8 | 39•4 | - 4•7 |
| 14/6/63 | 6•55 | 12•9 | 16 | - 3•45 | 14•3 | 29 | 39•4 | - 3•9 |
| 15/6/63 | 6•0 | 10•6 | 16 | - •6 | 14•4 | 26•5 | 39•4 | - 1•5 |
| 16/6/63 | 5•7 | 8•15 | 16•8 | + 3•0 | 12•1 | 27•2 | 39•4 | + 0•1 |
| 17/6/63 | 5•7 | 11•6 | 16•8 | - •5 | 14•1 | 32•4 | 39•4 | - 7•1 |
| 18/6/63 | 6•7 | 7•0 | 16•8 | + 3•1 | 12•8 | 25 | 39•4 | + 1•6 |
| 10 day Total + or - | | | | + 2•55 | Total | | | -17•3 |

Red Friesian 1963 Contd.

| | Sodium (gms./day). | | | | Potassium (gms./day). | | | |
|---|---|---|---|---|---|---|---|---|
| Date. | Faeces. | Urine | Intake | ± | Faeces | Urine | Intake | ± |
| 19/6/63 | 5•5 | 10•25 | 16•8 | + 1•05 | 15•6 | 26•5 | 39•4 | - 2•7 |
| 20/6/63 | 5•7 | 12•5 | 16•8 | - 1•4 | 13•5 | 25•4 | 39•4 | + 0•5 |
| 21/6/63 | 6•4 | 10•2 | 17•5 | + •7 | 13•2 | 25•8 | 39•4 | + 0•4 |
| 22/6/63 | 6•5 | 11•0 | 17•5 | 0 | 12•5 | 27•8 | 39•4 | - •9 |
| 23/6/63 | 6•2 | 15•5 | 17•5 | - 4•2 | 14•6 | 29•2 | 46•4 | + 2•6 |
| 24/6/63 | 6•0 | 15•3 | 17•5 | - 3•8 | 16•4 | 27 | 46•4 | + 3•0 |
| 25/6/63 | 6•1 | 15 | 17•5 | - 3•6 | 15•7 | 33 | 34•3 | +14•4 |
| 26/6/63 | 5•7 | 14•8 | 15•7 | - 4•8 | 16 | 28 | 45•8 | + 1•8 |
| 27/6/63 | 5•9 | 13•8 | 22 | + 2•3 | 13•1 | 32•8 | 41•3 | - 4•6 |
| 28/6/63 | 7•2 | 11•8 | 20•5 | + 1•5 | 11•1 | 25•7 | 29•8 | - 7•0 |
| 10 day Total + or - | | | | -12•35 | Total + or - | | | -21•3 |
| 29/6/63 | 6•4 | 22•4 | 13•8 | -15•0 | 9•2 | 27•7 | 24•1 | -12•8 |
| 30/6/63 | 4•8 | 9•6 | 14•1 | - •1 | 8•1 | 19•2 | 19•1 | - 8•2 |
| 1/7/63 | 4•2 | 15•1 | 9•8 | - 9•5 | 7•2 | 27•5 | 23•6 | -10•1 |
| 2/7/63 | 4•6 | 6•75 | 11•2 | - 0•15 | 6•8 | 26•5 | 8•6 | -24•7 |
| 3/7/63 | 3•4 | 2•69 | 4•8 | - 1•2 | 4•9 | 7•7 | 19•1 | + 6•5 |
| 4/7/63 | 2•5 | 3•1 | 7•9 | + 2•3 | 4•2 | 8•2 | 14•8 | + 2•4 |
| 5/7/63 | 3•2 | 3•9 | 17•5 | +10•4 | 5•4 | 12•7 | 28•9 | +10•8 |
| 6/7/63 | 4•3 | 10•25 | 10•3 | - 4•25 | 7•7 | 11•2 | 18•4 | - •3 |
| 7/7/63 | 4•9 | 9•6 | 12•7 | - 1•8 | 6•8 | 13•8 | 21•9 | + 1•3 |
| 8/7/63 | 4•5 | 8•1 | 12•7 | + •1 | 4•9 | 14•4 | 21•9 | + 2•6 |
| 10 day Total | | | | - 6•7 | Total | | | -42•5 |

Red Friesian 1963 Contd.

| | Sodium (gms./day). | | | | Potassium (gms./day) | | | |
|---|---|---|---|---|---|---|---|---|
| Date. | Faeces | Urine | Intake | ± | Faeces | Urine | Intake | ± |
| 9/7/63 | 4•2 | 5•6 | 3•1 | - 6•7 | 5•6 | 10•7 | 7•9 | - 8•4 |
| 10/7/63 | 3•1 | 3•5 | 3•1 | - 3•4 | 3•9 | 6•2 | 7•9 | - 2•2 |
| 11/7/63 | 2•9 | 3•7 | 8•4 | + 1•8 | 4•3 | 6•8 | 21•1 | +10 |
| 12/7/63 | 3•4 | 5•8 | 11•55 | + 2•35 | 9•5 | 10•3 | 28•65 | + 8•85 |
| 13/7/63 | 4•1 | 4•96 | 11•55 | + 2•49 | 7•8 | 15•6 | 28•65 | + 5•25 |
| 14/7/63 | 4•8 | 7•24 | 11•55 | - •49 | 10•5 | 16•2 | 28•65 | + 2•0 |
| 15/7/63 | 4•6 | 6•36 | 13•4 | + 2•44 | 12•8 | 18•1 | 33•15 | + 2•25 |
| 7 day Total + or - | | | | + 1•51 | Total + or - | | | + 7•75 |

Charollais I. Balance Experiment.

| | Sodium (gms./day). | | | | Potassium (gms./day). | | | |
|---|---|---|---|---|---|---|---|---|
| Date. | Faeces | Urine | Intake | ± | Faeces | Urine | Intake | ± |
| 5/3/64 | 3•40 | 10•0 | 15 | + 1•6 | 10•7 | 39•4 | 50•9 | + 0•8 |
| 6/3/64 | 3•2 | 9•0 | 15 | + 2•8 | 11•4 | 34•7 | 50•9 | + 4•8 |
| 7/3/64 | 3•65 | 6•85 | 15 | + 4•5 | 11•1 | 41•4 | 50•9 | - 1•6 |
| 8/3/64 | 3•5 | 8•53 | 15 | + 2•98 | 10•0 | 35•9 | 50•9 | + 5•0 |
| 9/3/64 | 2•97 | 7•9 | 15 | + 4•13 | 8•6 | 34•8 | 50•9 | + 7•5 |
| 10/3/64 | 4•10 | 9•55 | 13•3 | -00•35 | 9•1 | 29•9 | 56•6 | +17•6 |
| 11/3/64 | 5•6 | 5•75 | 12•5 | + 1•10 | 8•85 | 24•4 | 44•9 | +11•6 |
| 12/3/64 | 6•2 | 5•1 | 4•16 | - 7•14 | 7•3 | 25•2 | 22•7 | - 9•8 |
| 13/3/64 | 2•41 | 4•20 | 1•4 | - 5•21 | 4•92 | 45•8 | 23•5 | -27•2 |
| 14/3/64 | 1•58 | 2•59 | 3•7 | - 0•47 | 8•5 | 5•6 | 32•1 | +18•0 |
| 10 day Total + or - | | | | + 4•0 | Total + or - | | | +26•7 |
| 15/3/64 | 1•86 | 2•02 | 3•7 | - 0•18 | 15•4 | 16•8 | 32•1 | - 0•1 |
| 16/3/64 | 1•77 | 3•15 | 4•11 | - 0•81 | 13•4 | 17•3 | 40•2 | + 9•5 |
| 17/3/64 | 2•00 | 4•87 | 3•86 | - 3•01 | 12•8 | 17•3 | 30•1 | 0•0 |
| 18/3/64 | 2•41 | 4•10 | 4•11 | - 2•41 | 16•0 | 20•0 | 38•4 | + 2•4 |
| 19/3/64 | 1•88 | 4•2 | 4•11 | - 1•97 | 11•1 | 16•6 | 38•4 | +10•7 |
| 20/3/64 | 2•68 | 7•4 | 4•05 | - 6•03 | 10•2 | 27•8 | 31•7 | - 6•3 |
| 21/3/64 | 1•38 | 7•85 | 2•71 | - 6•53 | 6•85 | 26•8 | 36•0 | + 2•3 |
| 22/3/64 | 2•48 | 4•4 | 2•71 | - 4•17 | 10•6 | 33•0 | 36•0 | - 7•6 |
| 23/3/64 | 0•91 | 3•6 | 2•8 | - 2•75 | 9•4 | 29•5 | 4•9 | -34 |
| 24/3/64 | 1•19 | 2•71 | 2•71 | - 1•22 | 14•2 | 25•6 | 37•0 | - 2•8 |
| 10 day Total + or - | | | | -29•1 | Total | | | -25•9 |

Charollais Contd.

| | Sodium (gms./day). | | | | Potassium (gms./day ). | | | |
|---|---|---|---|---|---|---|---|---|
| Date | Faeces | Urine | Intake | ± | Faeces | Urine | Intake | ± |
| 25/3/64 | 1•36 | 3•78 | 2•73 | - 2•41 | 10•2 | 17•5 | 13•6 | -14•1 |
| 26/3/64 | 1•05 | 4•28 | 1•40 | - 3•93 | 6•15 | 25•1 | 2•45 | -28•8 |
| 27/3/64 | 0•28 | 0•91 | 5•52 | + 4•33 | 4•95 | 18•6 | 41•9 | +18•3 |
| 28/3/64 | 0•59 | 2•97 | 1•59 | - 1•97 | 6•44 | 18•9 | 10•5 | -14•8 |
| 29/3/64 | 0•59 | 4•5 | 1•59 | - 3•5 | 6•4 | 21•9 | 10•5 | - 7•8 |
| 30/3/64 | 1•12 | 4•85 | 2•84 | - 3•13 | 6•9 | 28•6 | 29•8 | - 5•7 |
| 31/3/64 | 0•57 | 0•75 | 2•35 | + 1•03 | 11•3 | 18•4 | 35•7 | + 6•0 |
| 1/4/64 | 0•69 | 0•66 | 2•35 | + 1•00 | 15•7 | 19•0 | 35•7 | + 1•0 |
| 2/4/64 | 1•00 | 1•49 | 2•68 | + 0•19 | 16•1 | 27•0 | 39•0 | - 4•1 |
| 3/4/64 | 1•26 | 0•90 | 2•68 | + 0•46 | 17•7 | 16•8 | 39•0 | + 4•5 |
| 10 day total + or - | | | | - 7•48 | Total + or - | | | -46•5 |
| 4/4/64 | 0•84 | 1•05 | 2•43 | + 0•54 | 13•3 | 20•8 | 35•1 | + 1•00 |
| 5/4/64 | 0•38 | 2•64 | 1•15 | - 1•87 | 6•60 | 13•2 | 2•37 | -17•4 |
| 6/4/64 | 0•49 | 1•11 | 1•78 | + 0•18 | 8•55 | 8•1 | 17•3 | - 3•4 |
| 7/4/64 | 0•34 | 1•10 | 3•10 | + 1•66 | 6•95 | 11•7 | 54•1 | +35•4 |
| 8/4/64 | 0•25 | 0•45 | 1•80 | + 1•10 | 6•90 | 15•8 | 18•1 | - 4•6 |
| 9/4/64 | 0•36 | 0•99 | 1•93 | + 0•58 | 6•78 | 12•2 | 18•4 | - 0•6 |
| 10/4/64 | 0•32 | 0•27 | 2•03 | + 1•44 | 7•40 | 12•2 | 25•2 | + 7•6 |
| 11/4/64 | 0•31 | 2•36 | 2•03 | - 0•63 | 10•3 | 8•45 | 25•2 | + 7•4 |
| 12/4/64 | 0•34 | 0•45 | 2•03 | + 1•24 | 11•5 | 13•5 | 25•2 | + 0•2 |
| 13/4/64 | 0•61 | 0•38 | 1•64 | + 0•65 | 12•2 | 12•2 | 13•7 | -10•7 |
| 10 day Total + or - | | | | + 4•9 | Total | | | +14•9 |

Charollais Contd.

| | Sodium (gms./day) | | | | Potassium (gms./day) | | | |
|---|---|---|---|---|---|---|---|---|
| Date. | Faeces | Urine | Intake | ± | Faeces | Urine | Intake | ± |
| 14/4/64 | 0·52 | 0·34 | 1·37 | + 0·51 | 10·6 | 8·1 | 6·8 | -11·9 |
| 15/4/64 | 0·61 | 0·93 | 1·37 | - 0·17 | 9·0 | 8·4 | 6·8 | -10·6 |
| 16/4/64 | 0·64 | 0·95 | 1·35 | - 0·24 | 8·74 | 10·5 | 9·1 | -10·1 |
| 17/4/64 | 0·75 | 1·36 | 1·25 | - 0·86 | 7·11 | 11·6 | 2·3 | -16·4 |
| 18/4/64 | 0·15 | 1·44 | 0 | - 1·59 | 1·11 | 7·5 | 0 | - 8·6 |
| 5 day Total + or - | | | | - 2·35 | Total + or - | | | -57·6 |

Friesian 1964. Balance Experiment.

| | Sodium (gms./day). | | | | Potassium (gms./day). | | | |
|---|---|---|---|---|---|---|---|---|
| Date. | Faeces | Urine | Intake | ± | Faeces | Urine | Intake | ± |
| 13/7/64 | 1•89 | 14•3 | 13•2 | - 3•0 | 5•24 | 39•2 | 45•9 | + 0•46 |
| 14/7/64 | 1•33 | 8•7 | 13•2 | + 3•2 | 4•04 | 37•8 | 45•9 | + 4•06 |
| 15/7/64 | 1•58 | 8•8 | 13•2 | + 2•8 | 6•75 | 34•2 | 45•9 | + 2•95 |
| 16/7/64 | 1•59 | - | 13•2 | - | 7•1 | - | 45•9 | - |
| 17/7/64 | 2•29 | 11•7 | 16•5 | + 2•5 | 7•65 | 37•2 | 45•9 | + 1•05 |
| 18/7/64 | 2•22 | 16•4 | 14•5 | - 4•1 | 4•9 | 30•8 | 45•9 | +10•2 |
| 19/7/64 | 2•96 | 11•1 | 14•6 | + 0•5 | 6•79 | 30•3 | 42 | + 4•91 |
| 20/7/64 | 2•71 | 11•5 | 14•6 | + 0•3 | 8•3 | 35•3 | 42 | - 1•6 |
| 21/7/64 | 2•84 | 11•9 | 14•6 | - 0•1 | 8•0 | 33•5 | 42 | + 0•5 |
| 22/7/64 | 4•68 | 10•8 | 14•6 | - 0•9 | 10•27 | 29•2 | 42 | + 2•53 |
| 10 day Total + or - | | | | + 1•2 | Total + or - | | | +26•66 |
| 23/7/64 | 4•32 | 12•2 | 14•6 | - 1•9 | 7•74 | 28•4 | 42 | + 5•86 |
| 24/7/64 | 3•26 | 9•6 | 14•6 | + 1•7 | 7•3 | 27•2 | 42 | + 7•5 |
| 25/7/64 | 3•22 | 7•9 | 14•6 | + 3•5 | 9•92 | 27•7 | 42 | + 4•38 |
| 26/7/64 | 4•27 | 10•8 | 14•6 | - 0•5 | 11•1 | 26•0 | 42 | + 4•9 |
| 27/7/64 | 3•62 | 10•6 | 14•6 | + 0•4 | 11•3 | 39•8 | 42 | - 9•1 |
| 28/7/64 | 3•91 | 11•5 | 14•6 | - 0•8 | 11•32 | 33•9 | 42 | - 3•2 |
| 29/7/64 | 3•76 | 10•3 | 14•6 | + 0•5 | 10•05 | 32•4 | 42 | - 0•45 |
| 30/7/64 | 2•88 | 11•6 | 14•6 | + 0•1 | 11•5 | 24•6 | 42 | + 5•9 |
| 31/7/64 | 3•63 | 12•2 | 14•6 | - 1•2 | 13 | 31•2 | 42 | - 2•2 |
| 1/8/64 | 3•17 | 14•0 | 14•6 | - 2•6 | 14•5 | 33•4 | 42 | - 5•9 |
| 10 day Total + or - | | | | - 0•7 | Total + or - | | | + 7•69 |

Friesian 1964 Contd.

| Date. | Sodium (gms./day). Faeces | Urine | Intake | ± | Potassium (gms./day). Faeces | Urine | Intake | ± |
|---|---|---|---|---|---|---|---|---|
| 2/8/64 | 3•12 | 10•1 | 14•6 | + 1•2 | 13•1 | 26•3 | 42 | + 2•6 |
| 3/8/64 | 3•20 | 13•5 | 14•6 | - 2•1 | 10•5 | 28•1 | 42 | + 3•4 |
| 4/8/64 | 2•79 | 11•2 | 14•6 | + 0•4 | 9•15 | 30 | 42 | + 2•85 |
| 5/8/64 | 2•75 | 13•2 | 14•6 | - 1•4 | 10•32 | 30•8 | 42 | + 0•90 |
| 6/8/64 | 3•64 | 13•3 | 14•6 | - 2•3 | 13•5 | 29•2 | 42 | - 0•7 |
| 7/8/64 | 2•82 | 10•3 | 13•2 | + 0•1 | 11•0 | 29•7 | 42 | + 1•3 |
| 8/8/64 | 2•37 | 10•7 | 13•2 | + 0•1 | 10•65 | 29 | 42 | + 2•35 |
| 9/8/64 | 1•74 | 12•5 | 13•2 | - 1•0 | 9•5 | 31 | 42 | + 1•5 |
| 10/8/64 | 1•77 | 12•2 | 13•2 | - 0•8 | 8•04 | 31•7 | 42 | + 2•26 |
| 11/8/64 | 1•80 | 12•9 | 13•2 | - 1•5 | 8•72 | 26•6 | 42 | + 6•68 |
| 10 day Total + or - | | | | - 7•1 | Total + or - | | | +23•0 |
| 12/8/64 | 1•59 | 9•3 | 13•2 | + 2•3 | 10 | 32•5 | 42 | - 0•5 |
| 13/8/64 | 1•39 | 6•6 | 13•2 | + 5•2 | 7•2 | 24•8 | 42 | +10 |
| 14/8/64 | 1•38 | 3•1 | 6•6 | + 2•1 | 7•5 | 23•1 | 21 | - 9•4 |
| 15/8/64 | 1•96 | 13•8 | 13•2 | - 2•6 | 8•3 | 28•0 | 42 | + 5•7 |
| 16/8/64 | 1•32 | 2•5 | 2•2 | - 1•6 | 8•0 | 10•8 | 7 | -11•8 |
| 17/8/64 | 0•80 | 2•3 | 3•6 | + 0•5 | 8•1 | 8•0 | 11•2 | - 4•9 |
| 18/8/64 | 1•14 | 2•7 | 3•2 | - 0•6 | 6•5 | 7•35 | 9•8 | - 4•1 |
| 19/8/64 | 1•36 | 3•2 | 1•9 | - 2•7 | 4•1 | 7•5 | 5•6 | - 6•0 |
| 20/8/64 | •822 | 1•9 | 1•1 | - 1•6 | 3•00 | 2•85 | 3•5 | - 2•4 |
| 9 day Total + or - | | | | + 1•0 | Total + or - | | | -23.4 |

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