METABOLITES IN URINE OF CATTLE WITH EXPERIMENTAL BLADDER LESIONS AND FED BRACKEN FERN [1]

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Summary

In attempts to detect the presence of urinary metabolites which may have a relationship to the cause of bovine enzootic hematuria, calves were fed a basal diet supplemented with fresh or dried bracken fern (*Pteridium aquilinum* (L.) Kuhn) and the urinary excretion of phenols, aromatic amines and fluorescent substances was compared qualitatively by paper chromatography with urines from calves receiving only the basal diet. In addition, quantitative analyses were made for a number of tryptophan metabolites, creatinine and 4-pyridoxic acid.

No consistent qualitative or quantitative differences were observed between the bracken-fed animals and contro s over a 13 month period. Neither were significant seasonal differences observed in either group. Clinical studies on these animals showed no change in blood cell counts or bleeding time. The four animals inoculated in the bladder with bovine cutaneous papilloma preparations developed tumor-like lesions in their urinary bladders.

INTRODUCTION

Enzootic bovine hematuria is a disease entity associated with neoplasia of the urinary bladder. It occurs in rather restricted areas of various parts of the world. The etiology is unknown although numerous factors have been suggested as the cause (Heeschen 1959). The association of neoplastic lesions in the urinary bladder has renewed interest in the etiology of this disease (Bretzinger 1957, Martincic 1953, Pamukcu 1955, 1957). Several workers associated bracken fern (*Pteridium aquilinum* (L.) Kuhn) (Fig. 1) with the occurrence of enzootic hematuria (Dilmen 1961, Götze 1942, Groh 1941, Hess 1950, 1951, Olson 1962, Pamukcu 1955, Rosenberger & Heeschen 1960, Sieber 1950) and this has received some support in feeding trials (Rosenberger & Heeschen 1960). Fig. 2 indicates the general world distribution of enzootic bovine hematuria.

Several of these areas have been visited by one (Olson) or two (Döbereiner and Olson) of us, and a low level of consumption of bracken fern by cattle has been observed (Figs. 3-7). A striking example of a relation between bracken fern and enzootic hematuria was noted in Brazil. A farmer moved to a farm with about 60 cattle, and two years later, the first case of enzootic hematuria occurred. During the next six years, 18 cattle developed the disease, 12 of which were sold and 6 of which died. He decided that improper nutrition was at fault and instituted his own remedial measures. These consisted of reducing the herd size so that there was more grazing area per animal, and some supplemental feed. Since his farm was about two miles from the road, and supplies and milk had to be carried this distance on pack mules and burros, the amount of supplemental feed was small. Bracken fern thrives in pasture areas and overgrows the grasses normally eaten by cattle, causing them to consume bracken fern for sustenance. In this area of Brazil it has been customary for farmers to cut the bracken plants in pastures once or twice a year, but new plants develop from the roots. This farmer developed the practice of cutting bracken at least four times a year. The contrast of grass in his pasture with that of a neighbor's pasture was marked (Fig. 8). During the next seven years on this regimen, enzootic hematuria ceased to be a problem on this farm, although it has continued to be a problem with neighbors. This farmer, acting on his own initiative and resourcefulness, has provided a practical experiment, the results of which, incriminate low level intake of bracken fern in the etiology of enzootic hematuria. The disease has essentially disappeared in many areas of the world where it was

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FIG. 1. *Bracken fern growing in cattle pasture of northern Honshu island, Japan. A few cases of enzootic hematuria occurred in cattle kept on this pasture.*

with spontaneous bladder cancer excreted excess aromatic amines in their urine. This was further supported by the observations that a high incidence of urinary bladder tumors resulted when rats were fed 2-acetylaminofluorene combined with tryptophan (Dunning *et al.* 1950) or indole (Dunning & Curtis 1958), but failed to develop bladder cancer when fed 2-acetylaminofluorene alone. Further, in human clinical studies it was found that approximately half of the patients with spontaneous carcinoma of the bladder excreted elevated amounts of aromatic amines and aminophenols derived from tryptophan (Boyland & Williams 1955, 1956, Brown *et al.* 1955, 1960). In cases of schistosomiasis without bladder cancer there was a twofold increase in 3-hydroxyanthranilic

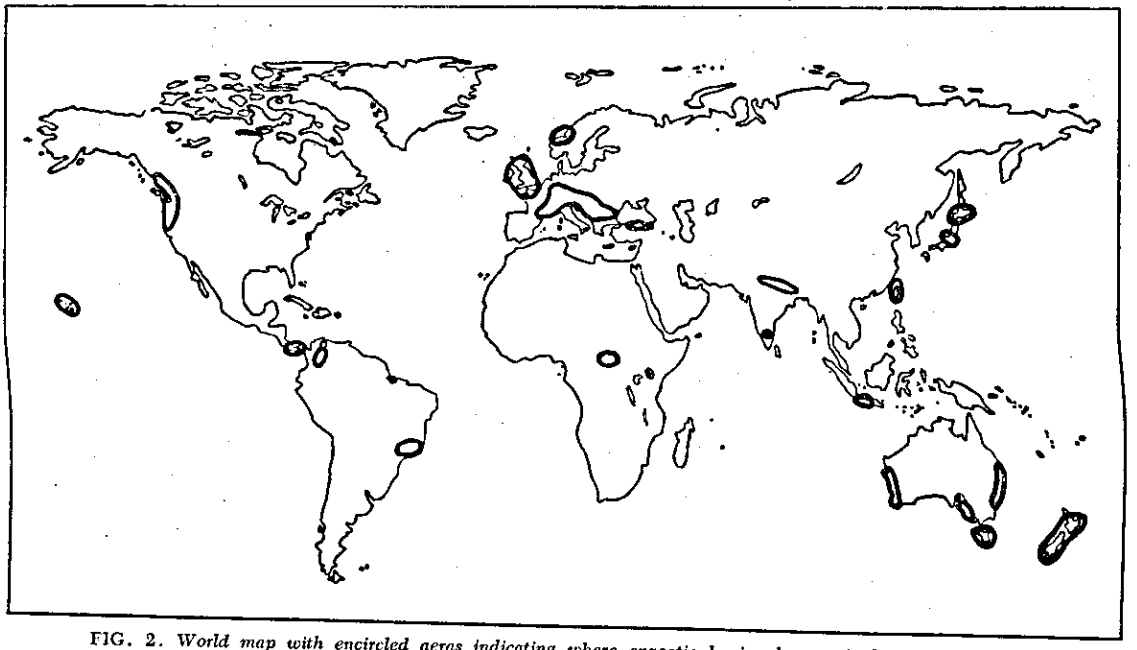

FIG. 2. *World map with encircled aeras indicating where enzootic bovine hematuria has been reported.*

once a major problem, for example on the Pacific northwest coast of North America, certain regions of Australia, New Zealand, Scotland, and Hawaii. Improved farming practices are usually suggested as the reason for the decreased incidence of the disease.

The urogenous route of chemical carcinogenesis of the bladder was demonstrated by the use of bladder pouches (McDonald & Lund 1954) or by transplantation of the ureters (Scott & Boyd 1953) in dogs prior to feeding 2-napthylamine. Tumors arose only in those dogs in which the bladder was in contact with urine.

That tryptophan or its metabolites might be involved in bladder carcinogenesis was suggested by the report (Ekman & Strömbeck 1947) that patients

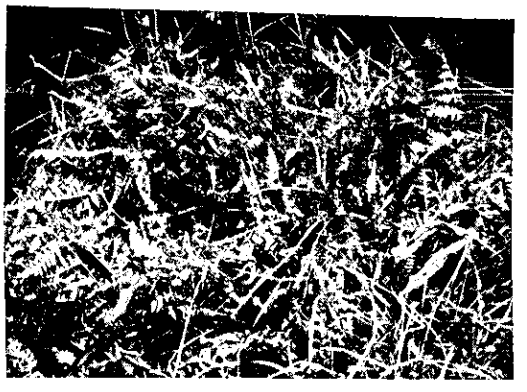

FIG. 3. *Hay containing about 15 to 20 per cent of bracken fern. This was to be fed to cattle in an area of Japan where enzootic hematuria occurs.*

FIG. 4. *Bracken fern in a cattle pasture of Japan. Only a few cases of enzootic hematuria occurred in cattle kept on this pasture. There is an ample supply of grass so that cattle are not forced to eat the fern.*

acid in the urine, but a fourfold increase in cases of schistosomal cancers of the bladder (Abul Fadl & Khalafallah 1961). Price and Brown (1962) found that patients with bladder cancer due to exposure to industrial aromatic amines excreted normal levels of kynurenine and hydroxykynurenine in contrast to abnormal levels found in patients with spontaneous bladder cancer, or several other disease conditions (Price 1958). The observations that several urinary tryptophan metabolites are carcinogenic for the urinary bladder of mice (Boyland & Watson 1956, Allen *et al.* 1957, Bryan *et al.* 1963) lends support to the hypothesis that normal metabolic products of tryptophan might be involved in spontaneous bladder carcinogenesis.

Dann (1962) made rather extensive studies on enzootic hematuria in Australia during the past 20 years. Unfortunately, the details of these studies have not been published. He found higher values for free and for combined phenols in urine from affected animals. The phenolic acid fractions from urine of affected animals gave absorption spectra which in most cases could result from mixtures of p-hydroxybenzoic, p-hydroxyphenylacetic and p-hydroxyphenyl propionic acids, but in addition, normal urine appeared to contain another component, probably a diphenolic acid, only traces of which occurred in affected urine. Paper chromatography was unsuccessful in identification of the compound. Apparently significant differences were found in the aromatic amines but various attempts to further study these amines were met with technical difficulties. The work of Dann was somewhat similar to studies on excretion of aminophenols and tryptophan metabolites being pursued by other laboratory groups working with the bladder tumor problem.

Pamukcu *et al.* (1959) compared the excretion of tryptophan metabolites in urine from Turkish cows having bladder tumors with metabolites in urine from normal cattle of the same area. Only acetylkynurenine was found to be significantly higher in the urine of tumor bearing animals.

The duration of the disease, varying from two months to six years, had no effect upon the level of

FIG. 5. *Bracken fern in pasture on Fiji islands with an abundance of other grasses. No cases of enzootic hematuria have been reported from this area.*

urinary excretion of these metabolites. That greater differences between the two groups were not found may be due to the fact that both groups of cows were from the same area and were eating similar forage. Normal cattle in Wisconsin, where the incidence of bladder tumors is low, generally excreted greater quantities of tryptophan metabolites than Turkish cattle (Pamucku *et al.* 1959).

This report compares observations on certain metabolic constituents in urine from cattle fed bracken fern with those in urine from cattle not fed bracken fern. No differences were noted under the conditions of these studies. In view of the possible stimulatory effect of bracken fern metabolites on bladder lesions produced by inoculation of bovine cutaneous papilloma agent (Olson *et al.* 1959, 1962) such bladder lesions were induced in some of the animals for observation during the feeding of bracken fern. This phase of the study is still in progress.

FIG. 6. *Cow eating bracken fern on a farm in Municipio Santo Antonio, Minas Gerais, Brazil. On this farm, 5 to 6 cases of enzootic hematuria occur each year in a herd of about 40 adult cattle. Symptoms are first noted when cattle are 3 to 5 years old.*

FIG. 7. *Fronds of bracken fern have been stripped from the stem of the plant by cattle eating the fern. On same farm as Fig. 6.*

FIG. 8. *The hill in the background has a fence separating the pasture on the right, in which bracken fern was controlled by cutting 4 to 5 times per year, from the pasture on the left, in which there is considerable growth of bracken even though cut once a year. No cases of enzootic hematuria occur in the pasture on the right which is relatively free of bracken. The disease was a problem in cattle kept on the other pasture in which bracken fern is evident.*

MATERIAL AND METHODS

Cattle. One male (107) and six female Holstein calves were used in this experiment. They were approximately one to three months old when obtained from farms said to be free of bovine papillomatosis.

Preparation of urinary bladder pouches and inoculations. Surgical operations of the urinary bladder were made in all animals, se that each animal had a bladder pouch. This pouch isolated about one third of the bladder mucosa from the f'ow of urine in the bladder proper. A second operation was performed in all animals except animal 104 in order to inoculate papilloma materials. Both the bladder pouch and bladder proper were opened and inoculated in the submucosa. Animals 105, 107, 108 and 109 were inoculated with bovine cutaneous papilloma material (isolate 260); animals 110 and 112 were inoculated with material obtained from bladder lesions of a case of enzootic hematuria in British Columbia, Canada (isolate 200). Each animal receiving a bladder inoculation was also inoculated in the skin with the same material at the same time.

Bracken fern feeding. All animals received the same basic diet of timothy and alfalfa hay and a standard dairy calf feed supplement (1% salt, 27% bran, 27% corn, 27% oats, and 18% linseed oil meal).

Four weeks after the last surgery, each of four calves (108, 109, 110 and 112) received a daily bracken hay supplement of approximately 1.4 to 2.9 g/kg body weight or, according to the season of the year, a daily supplement of fresh green bracken of approximately 3.3 to 4.3 g/kg body weight. The bracken fern was harvested in central Wisconsin, in August 1961 and from July to September 1962. There was a history of acute bracken poisoning of cattle in these areas. Mature and green plants were cut near the base of the stem. The bracken collected in 1961 was dried artificially in a hay dryer at 44° to 49°C. Most of the harvest of 1962 was sun dried. Some of the bracken fed in a fresh green state was stored for a few days prior to feeding at approximate'y 4°C. All the bracken was chopped in a hammer mill.

Animals 104, 105 and 107 did not receive bracken and served as control animals.

Blood cell counts and bleeding time. From time to time blood cell counts were made and bleeding time was recorded in order to detect a possible depression of bone marrow actitivity, an early indication of acute bracken poisoning (Evans *et al.* 1954).

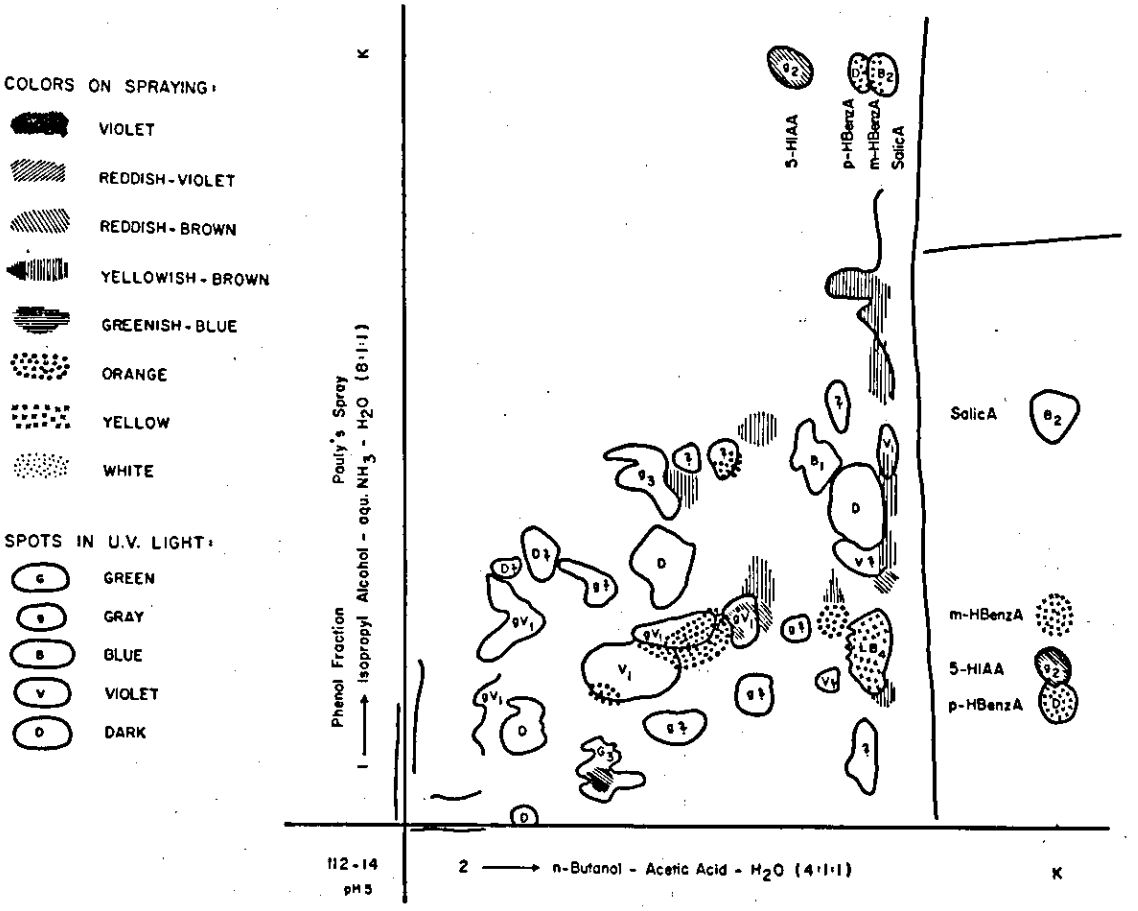

FIG. 9. *Typical two-dimensional paper chromatogram from urine extract of bracken fed calf. Qualitatively similar chromatograms were observed with control specimens from animals not fed bracken fern. Known reference compounds, applied at K were salicylic acid (SalicA), m-hydroxybenzoic acid (m-HBenzA), 5-hydroxyindoleacetic acid (5-HIAA), and p-hydroxybenzoic acid (p-HBenzA). Numerical subscripts to the ultra-violet fluorescence symbols designate relative intensity of fluorescence on a scale from 1 to 5 with 1 designating the least detectible amount of fluorescence.*

Examination of the urinary bladder. The urinary bladders of the female experimental animals were examined with a cystoscope for presence of lesions in connection with another phase of work to be reported later.

Urine collection and routine urine analyses. Urine samples were collected every month while the animals were urinating naturally. Small portion of the urine were examined shortly after collection and samples for paper chromatography and analyses with ion-exchange columns were preserved at -8°C after addition of 1% glacial acetic acid and 2% toluene. Routine analyses of fresh unpreserved specimens included pH measurement with a Beckman glass electrode potentiometer, test for albumin with Robert's reagent method (Coffin 1953), macroscopic and microscopic examination of the sediment and the benzidine test for hemoglobin.

Paper chromatography of urine samples. Twenty-one urine samples collected at intervals throughout the study were analyzed by paper chromatography. Methods developed by Dalgliesh (1955) and by Armstrong *et al.* (1955) were adapted for semiquantitative examination of these samples. Since the cow, like many other animals, excretes creatinine at a constant rate (Groot & Aafjes 1960), the creatinine content of the urine was used as a measure for the concentration of urinary constituents. Creatinine was determined as described by Peters (1942).

Chromatographic analyses of the urine samples collected from the experimental animals was as follows: A quantity of urine corresponding to 50 mg creatinine was mixed with 4 g active charcoal. The charcoal was filtered off from the urine sample, washed with distilled water, and eluted by 200 ml of

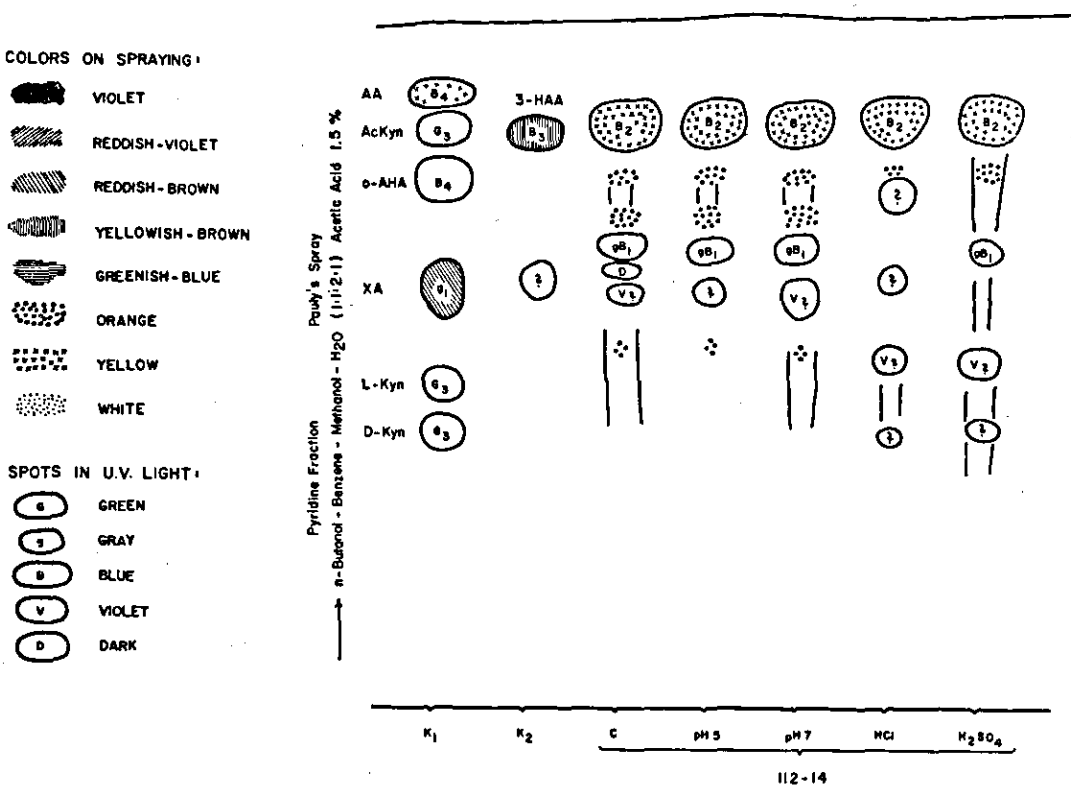

FIG. 10. *Typical one-dimensional chromatogram of pyridine eluates of charcoal extracts of urine from a bracken fed calf. Qualitatively similar chromatograms were observed with specimens from animals not fed bracken fern. Known reference compounds applied at K1 and K2 were anthranilic acid (AA), acetylkynurenine (AcKyn), o-aminohippuric acid (p-AHA), xanthurenic acid (XA), L-kynurenine (L-Kyn), D-kynurenine (D-Kyn) and 3-hydroxyanthranilic acid (3-HAA). C referes to the unhydrolized sample while pH 5 and refer to samples hydrolized with B-glucuronidase at these pH values; HCl and H_2SO_4 refer to samples hydrolized by these acids as described in the text.*

an aqueous solution of 8% phenol. A second fraction was obtained by eluting the same charcoal with 50 ml of an aqueous solution of 5% pyridine. The two eluates were evaporated in a water-bath at 40° to 50° C under reduced pressure. The concentrates were washed from the flask with 50% ethyl alcohol and again dried in beakers. Four ml of 50% ethyl alcohol were added to each fraction and aliquots were applied to the paper with micro-pipettes. A quantity of concentrate (20 μ liter) equivalent to 0.25 mg creatinine, applied in one spot on Whatman No. 1 paper, gave the best separation on the chromatogram.

The solvent system used for one-dimensional chromatography of phenol and pyridine fractions of the urine samples was methanol, butanol, benzene, and water (2:1:1:1), as described by Mason and Berg (1951) but containing 1 ml of glacial acetic acid per 100 ml of the solvent. The solvent systems used for two-dimensional chromatograms of the phenol fractions were isopropyl alcohol, ammonia and water (8:1:1) for the development in the first dimension for 16 hours, and n-butanol, acetic acid and water (4:1:1) for the development in the second dimension for 9 hours.

Reference standards such as anthranilic acid, 3-hydroxyanthranilic acid, kynurenine, acetylkynurenine, o-aminohippuric acid and xanthurenic acid, were run simultaneously on each one-dimensional chromatogram. The standards for two-dimensional chromatograms were salicylic acid, m- and p-hydroxybenzoic acid and 5-hydroxyindole-3-acetic acid. The properties or reactions used for the examination of the chromatograms were: Fluorescence under ultraviolet light, Ekman's reaction (Ekman 1948) and Pauly's reaction (Dalgliesh 1955).

Known amounts of acetylkynurenine, o-aminohippuric acid, 3-hydroxykynurenine, kynurenine and anthranilic acid were added to a urine sample to test the recovery of known quantities of these aromatic amino compounds by the charcoal method. With the

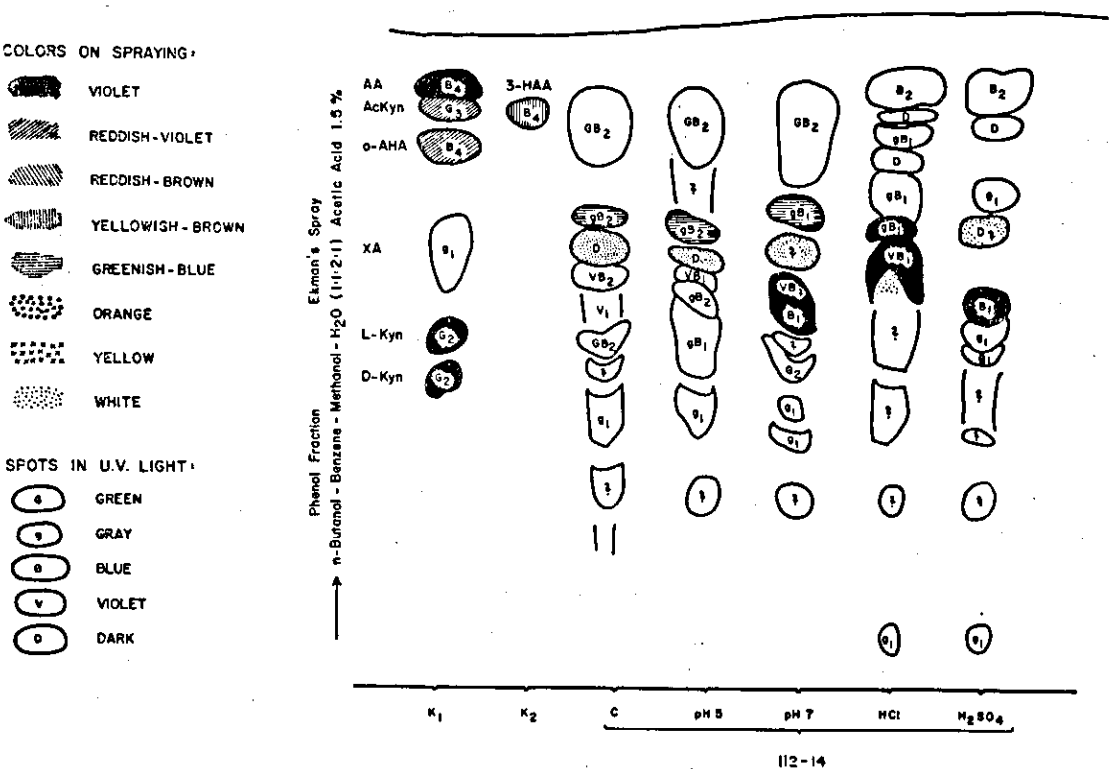

FIG. 11. *Typical one-dimensional chromatogram of phenol eluate of charcoal extracts of urine from bracken fed calf. The abbreviations are the same as described in Fig. 10.*

aliquots used (0.25 mg creatinine equivalents), a recovery of 100% corresponded to 10 μg of each compound.

Some urine samples were hydrolyzed by one or more of four hydrolytic agents which were mammalian (bovine liver) beta-glucuronidase, bacterial beta-glucuronidase, hydrochloric acid and sulphuric acid. Urine samples at pH 5 and pH 7 were incubated at 38° C for about 18 hours with 40 units per ml of the beta-glucuronidase (Sigma Chemical Company, St. Louis, Mo.). The buffering of these samples was carried out by dissolving the calculated amounts of citric acid and sodium biphosphate (McIlwain 1921) in 50 ml of urine. The method of Sperber (1948) was used to hydrolyze urinary ethereal sulphates with hydrochloric acid, and the method of Fishman and Green (1955) was used to hydrolyze the conjugated urinary beta-glucuronides with sulphuric acid. Hydrolysis with hydrochloric and sulphuric acid was carried out at 100° C for 90 minutes, which was found to be sufficient for complete hydrolysis.

In order to improve separation of the phenolic fractions from urine incubated with bacterial beta-glucuronidase, the acidic solvent of Mason and Berg (1951) was used for development in the first dimension and n-butanol-acetic acid-water solvent (4:1:1) for development in the second dimension. The material, in these cases, was applied to the paper in a concentration corresponding to 0.625 mg creatinine.

Quantitative analysis for urinary metabolites. Quantitative analyses were done for fraction A, anthranilic acid glucuronide, o-aminohippuric acid, acetylkynurenine, kynurenine, and hydroxykynurenine using methods described previously (Brown 1957, Brown & Price 1956). In addition, kynurenic acid, and xanthurenic acid (Satoh & Price 1958), N-methyl-2-pyridone-5-carboxamide (Walters *et al.* 1955), N'-methylnicotinamide (Vivian *et al.*), and 4-pyridoxic acid (Reddy *et al.* 1958) were measured. All analytical data were calculated as mg per gram urinary creatinine to compensate for individual variations in urine dilution (Groot & Aafjes 1960).

RESULTS AND DISCUSSION

The animals fed bracken developed no signs of poisoning. Bleeding time and counts of erythrocytes

and thrombocytes of the bracken fed and of the control animals were normal and not essentially different from each other. Counts of the leucocytes of both groups were higher than normal, principally in the control animals. This could be related to lesions caused by trauma during cystoscopy. Differential counts made near the end of the experimental period were essentially similar in control and bracken fed animals and to the values given by Schalm (1961).

The observations indicated a reaction from inoculations in only four animals. Tumor growth in the urinary bladder developed in the four animals (105, 107, 108 and 109) inoculated with bovine cutaneous papilloma agent (isolate 260). The growth in the urinary bladder was characterized by polyp formation. These were similar to those previously described (Olson *et al.* 1959, 1962). Palpation of the bladder pouches gave evidence for tumor growth in two of these cases. Warts on the skin were observed at the site of inoculation in the four calves with bladder lesions. The inoculation of the enzootic hematuria material (isolate 200) did not result in any tumor growth on the skin or the urinary bladder of two calves. Bracken fern intake under the experimental conditions had apparently no effect on the development of urinary bladder tumors caused by the inoculation of bovine cutaneous papilloma material.

No marked hematuria was noted in the experimental animals during 13 months of the feeding trial. The red and white blood cells found in the urine by microscopic examination were apparently due to the bladder lesions caused by surgery, inoculation and cystoscopy and were just as frequent in the control as in the bracken fed animals. In some instances the presence of red blood cells in the urine could be attributed to metrorrhagia during estrus. The occasionally opaque or slightly flocculent appearance of the fresh voided urine samples was correlated with the presence of cellular elements in the urnie. The benzidine test for hemoglobin gave weakly positive results only when red blood cells were readily found in the urine by microscopic examination. The pH of the urine samples was generally alkaline. Albumin was found in the urine many times and seemed to be related to the bladder lesions.

About 75 paper chromatograms prepared from phenol and pyridine fractions of 21 urine samples were developed in one or two dimensions and examined under ultraviolet light. Some of these papers were sprayed with Ekman's reagent for diazotizable aromatic amines, and most of them with Pauly's reagent for phenols and compounds capable of coupling with diazotized sulfanilic acid. No qualitative differences with regard to diazotized sulfanilic acid were found between the urine samples of the bracken fed and control animals, and the results presented in Figs. 9 to 11 are representative of those seen in both groups of animals. However, minor and inconstant quantitative differences could be traced on the paper chromatograms. The recovery of known quantities of several aromatic amino compounds on the chromatogram was approximately 50% by the method used. It was thought that more of these metabolites could be detected by hydrolysis of urine samples before filtering through active charcoal. The hydrolysis of the urine modified the number and distribution of unidentified spots on the paper (Figs. 10 and 11), but there were no obvious differences in the chromatograms of the urine from animals fed bracken and control animals. The analytical methods may not have been suitable for the detection of minor differences in the excretion of urinary metabolites by these animals, or the quality and amount of bracken fed may not have been adequate to produce detectible differences.

The average quantitative analyses for tryptophan metabolites and other urinary products over a 13 month period are presented in Table 1. They show no differences between control animals and those fed bracken fern. In addition, the data were analyzed for seasonal variations within these groups and none were observed.

The feeding of bracken fern to some of the experimental animals was continued and another approach to the question of carcinogens in the urine has been made by Pamukcu, Alson and Price[5]. The procedure was to extract the urine with ethyl acetate and divide it into acidic and non-acidic fractions.

TABLE 1. Average quantitative excretion of metabolites by calves fed control and bracken supplemented diets [a]

Metabolites in urine	Bracken fed	Non-bracken fed
Kynurenic acid	3.2 ± 1.5	3.2 ± 1.5
Xanthurenic acid	3.9 ± 2.3	3.6 ± 2.3
"A" fraction	218 ± 6.5	195 ± 2.2
Anthranilic acid glucuronide	7.7 ± 1.8	7.4 ± 6.0
o-Aminohippuric acid	13.2 ± 3.0	11.9 ± 3.8
Acetylkynurenine plus anthranilic acid	5.4 ± 1.5	4.8 ± 2.6
Kynurenine	7.3 ± 2.1	9.0 ± 2.9
Hydroxykynurenine	4.1 ± 2.3	5.2 ± 4.5
N1-methylnicotinamide	19.9 ± 6.1	22.4 ± 7.1
N-methyl-2-pyridone-5-carboxamide	4.0 ± 2.6	3.8 ± 3.5
4-Methyl-2-pyridone-5-carboxamide	2.42 ± 0.73	2.32 ± 0.9
4-Pyridoxic acid	1.07 ± 0.26	0.9 ± 0.18
g creatinine/liter		

[a] Values are expressed as mg/g creatinine ± standard deviation. The number of analyses for each metabolite varied 39 and 53, and specimens were taken over a 13 month period from three control and four bracken fed calves.

[5] Assay of fractions of bovine urine for carcinogenic activity after feeding bracken fern (*Pteris aquilina*). Manuscript in preparation.

The solvent was removed from the fractions and the residue combined with cholesterol to form pellets. The pellets were then placed in the lumens of urinary bladders of mice to test the prolonged exposure of bladder epithelium to the residues of the extracts from cow urine. Five of 15 mice exposed to the acidic fraction of the urine from cattle fed bracken fern developed carcinoma in their urinary bladders. While only one of 13 mice exposed to a comparable fraction from urine of control cows not fed bracken fern. These preliminary results suggest that a carcinogen is present in the urine of cattle fed bracken fern, thus confirming the circumstances observed on farms where the disease occurs. The failure to detect significant increase of tryptophan metabolites as a result of feeding bracken fern may mean that another type or group of carcinogenic compounds should be sought in the urine.

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SUBSTÂNCIAS METABÓLICAS NA URINA DE BOVINOS COM LESÕES EXPERIMENTAIS DA BEXIGA E QUE RECEBERAM SAMAMBAIA (*Pteridium aquilinum* (L.) Kuhn) NA SUA ALIMENTAÇÃO

Sumário

Na tentativa de verificar a presença de substâncias metabólicas na urina que podiam ser relacionadas com a causa da hematuria enzoótica dos bovinos, foi realizado um experimento em bezerros que receberam com sua alimentação básica um suplemento de samambaia (*Pteridium aquilinum* (L.) Kuhn) em estado verde fresco e como feno. Através de cromatografia de papel comparou-se qualitativamente o teor de fenois, de aminas aromáticas e de substâncias fluorescentes na urina dêstes bezerros, com o teor destas substâncias na urina de bezerros testemunhas que foram alimentados sòmente pela ração básica. Adicionalmente foram feitas análises quantitativas de uma série de substâncias metabólicas oriundas do triptofano, bem como análises de creatinina e de ácido 4-pirodóxico.

Não foram observadas diferenças qualitativas ou quantitativas constantes entre os animais que receberam o suplemento de samambaia e o grupo testemunha durante um período de 13 meses. Também não foram verificadas em cada grupo diferenças significativas em relação a estação do ano. Estudos clínicos não revelaram alterações na contagem de elementos sangüíneos ou no tempo de coagulação. Os quatro bezerros inoculados na bexiga com preparações de papiloma cutâneo desenvolveram lesões tumoriformes neste órgão.