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Metabolites of riboflavine in milk, urine and tissues of animals in relation to alimentary symbiotic bacteria

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1. Riboflavine given orally to sheep, calves, goats and ruminating kids gave rise, in milk and urine, to extra riboflavine accompanied by hydroxyethylflavine, by formylmethylflavine and by other as yet unidentified metabolites, but subcutaneous injection of riboflavine caused prolonged excretion of riboflavine in urine and milk unaccompanied by metabolites.

2. Placement of riboflavine by means of fistulas into the rumen, the abomasum or the caecum and abstraction of samples from the rumen or caecum showed that the rumen was the site of origin of the metabolites, thus accounting for the lapse of time of several hours between the ingestion of riboflavine and the appearance of hydroxyethylflavine in the urine and the absence of metabolites after subcutaneous injection of riboflavine.

3. Kids in which rumination had not yet become established could not produce metabolites from ingested riboflavine although, when fed milk containing the metabolites, they readily excreted them in their urine.

4. After injection of formylmethylflavine subcutaneously into a kid, its urine was found to be fluorescent from the presence of formylmethylflavine and its reduction product, hydroxyethylflavine. After formylmethylflavine was injected into a milking goat it appeared in both milk and urine accompanied by hydroxyethylflavine.

5. Incubation in vitro of kidney or liver tissue from cattle or goats with riboflavine did not give rise to metabolites, but these tissues could both reduce formylmethylflavine to hydroxyethylflavine in vitro.

6. Post-mortem examination of animals to which riboflavine had been fed showed the presence of metabolites only in gut contents, in the urinary bladder and in the bile. When injected, riboflavine reached the bile but no metabolites were produced.

7. Metabolites were found to occur naturally in the urine of ruminants in small amounts. Hydroxyethylflavine was readily demonstrable in the urine of the rabbit, but in the urine of the dog metabolites could not be found.

8. From these and earlier and later experiments the conclusion is drawn that riboflavine metabolites arise in the ruminant from the degradation of riboflavine by rumen bacteria.

For non-ruminants such as man, pig, cat, dog, rat or birds, riboflavine (vitamin B_2) is a dietary essential. Adult ruminants, however, benefit from the activities of the micro-organisms in the rumen (Owen, 1967). After weaning, young ruminants rapidly acquire characteristic microflora and microfauna in the rumen, which enable them to develop an independence of exogenous sources of essential amino acids and B-vitamins (Edwards & Darroch, 1956; Crossland, Owen & Proudfoot, 1958; Virtanen, 1967).

The daily requirement of an animal for riboflavine can be measured in mg/100 kg body-weight, but in our experience doses of 5 g to animals weighing 40 kg (125 μg/g) produce no toxic effects. Fox & Miller (1960) found no ill effects in rats receiving injections of 120 μg/g body-weight. Such an injection would be equivalent to 9 g riboflavine for a 75 kg man. On several occasions members of the staff of this department, including the authors themselves, have eaten as much as 1 g riboflavine without any effects other than the production of urine which fluoresced greenish yellow in u.v. light (Owen & Dzialoszynski, 1965*a*).

When eaten by animals or man in quantities (100 mg to 1 or more g) much larger than a normal daily requirement riboflavine considerably increases the urinary riboflavine while at the same time causing the appearance in the urine of various metabolites which were first identified in this laboratory (Owen, 1962*a*, *b*). We have identified two of these metabolites of riboflavine as 7,8-dimethyl-10-(2′-hydroxyethyl)-isoalloxazine (hydroxyethyl-flavine) (HEF) (Owen, 1962*a*, *b*) and 6,7-dimethyl-10-(formyl-methyl)-isoalloxazine (FMF) (Owen & West, 1968*a*, *b*) but there are a number of others in both milk and urine which remain to be identified. In recent

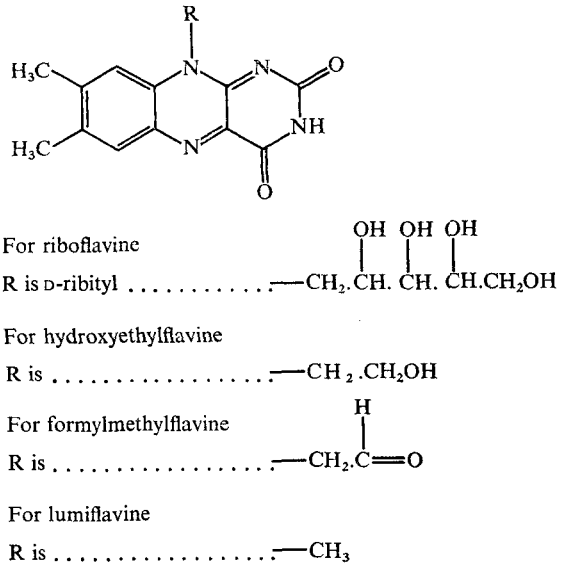

Fig. 1. Riboflavine and related isoalloxazines.

experiments (West & Owen, 1969) we have found HEF, FMF (Fig. 1) and other as yet unidentified metabolites accompanying riboflavine in the urine of man after ingestion of riboflavine.

Certain species of rumen bacteria can degrade riboflavine (Hobson, Summers, Owen, Spencer & West, 1969) and we believe it is these bacteria which give rise to the metabolites which are excreted in the urine and secreted in the milk when excess riboflavine is fed. To investigate this hypothesis we have administered riboflavine to animals by mouth, by fistula into various parts of the gut and also by subcutaneous injection. In addition we have compared the metabolic fates of HEF, FMF and riboflavine in young and older ruminants. Contents from the rumen and other parts of the gut have been incubated in vitro with added riboflavine and in vitro studies have been made of kidney and liver tissue incubated with riboflavine or with FMF. The present paper is a report of the results of these studies.

EXPERIMENTAL AND RESULTS

Detection of isoalloxazines in biological materials

In solution or on chromatograms riboflavine and its metabolites may be detected by their yellow fluorescence in long-wave u.v. light. Originally we used paper chromatography for both detection and isolation of metabolites but we now prefer preparative and thin-layer chromatography on silica gel ('Kieselgel' D5; Camag, Switzerland). Urine can be chromatographed on thin layers without preliminary extraction, but milk must be deproteinized with trichloroacetic acid (Bessey, Lowry & Love, 1949). We have found that some systems of chromatography in common use fail to distinguish HEF from lumiflavine (Fig. 1). However, these compounds can be distinguished by partition methods, HEF being extractable by dilute acid from chloroform (Owen, 1962*a*) and lumiflavine being preferentially soluble in chloroform (Sone, 1958). In addition, HEF, but not lumiflavine, forms an acetoxy-derivative when treated in the cold with acetic anhydride in pyridine solution (Owen, Montgomery & Proudfoot, 1962). Acetoxy-HEF has a different R_F in most systems from that of HEF or lumiflavine. We therefore routinely convert chromatographically isolated HEF into its acetoxy-derivative to confirm its identity with authentic material perpared by using the method of Fall & Petering (1956). Acetoxy-HEF is extractable from aqueous solution by chloroform and is recognizable by microscopy of its crystals in polarized light (E. C. Owen, to be published), or by other physicochemical means (Owen & West, 1968*a*, *b*).

If a newly isolated, yellow-fluorescing substance is an isoalloxazine it will have a typical four-banded absorption spectrum with peaks at or close to 220, 270, 350 and 445 nm that show characteristic changes with pH (Cresswell & Wood, 1960; Owen, 1962*a*). The ratio E_{max} 445 nm : E_{max} 272 nm increases to about one-third as the colourless u.v.-absorbing substances commonly present in biological systems are removed during purification (Owen, 1962*a*, *b*).

Strips of paper or preparative layers were eluted by the method of Owen (1968). The paper strip was rolled up inside a centrifuge tube with a perforated base. For elution from silica gel a plug of glass wool covered with a layer of Celite (Johns, Manville and Co., London) prevented silica from reaching the eluate. This tube was suspended, coaxially, by means of a flanged washer, within a larger one. In this apparatus residual chromatographic solvents were removed by centrifugation with diethyl ether in which the metabolites are insoluble. Two to five centrifugations with an appropriate solvent then completely extracted the isoalloxazines, which could be measured either fluorimetrically or microbiologically.

Quantitative assay of isoalloxazines

Riboflavine: microbiological assay. In the *Lactobacillus casei* (ATCC 7469) assay, the metabolites of riboflavine and many derivatives of isoalloxazine, e.g. galactoflavine, can spare riboflavine but cannot replace it. This sparing effect is however only a fraction of the requirement (Lane, Petering & Brindley, 1959; Owen & Spencer, unpublished). In large excess over riboflavine, these unphysiological isoalloxazines

inhibit the growth of *L. casei*. In urine and milk obtained after large or small oral doses of riboflavine, the concentrations of non-riboflavine isoalloxazines are such that the assay gives a measure of the true riboflavine present. Bacterial growth was measured from turbidity in the Unicam SP 500 at 600 nm (Lane *et al.* 1959).

Isoalloxazines (including riboflavine) in eluates from chromatograms. These were determined by measurement of their fluorescence as described by Crossland *et al.* (1958), but the Locarte Mark IV fluorimeter. The blue primary filter LF 3 was used to allow the 435·8 nm line of the mercury vapour lamp to illuminate the sample. To allow the fluorescence so generated to reach the photomultiplier tube, the filter LF 7 was used in the secondary path. Together these two filters block all the visible spectrum, so minimizing any access of scattered light to the photomultiplier. The activation spectrum of riboflavine has a marked peak at 470 nm (Lane, Fahey, Sullivan & Zubrod, 1958) and the 435·8 nm line, one of the strongest in the mercury spectrum, falls on a shoulder of this 470 nm peak at a point where it has about four-fifths of its maximum intensity. The fluorescence spectrum of riboflavine has a single peak at 520 nm (Lane *et al.* 1958) the light from which passes filter LF 7. Most of our measurements of fluorescence have been made in water, but other solvents may be used. After readings in aqueous solution the fluorescence of the isoalloxazines can be quenched by adding dithionite and bicarbonate to measure any non-quenchable blank. Such blanks were usually small. A calibration curve was prepared by dilutions of a standard solution of riboflavine.

Quantitative preparative layer chromatography of isoalloxazines

Glass plates (20 × 30 cm) were spread with a 0·75 mm layer of the suspension of silica gel, allowed to dry overnight before being heated in an oven at 90° for 1 h, and then left to cool to room temperature. Urine (0·15 ml) was applied as a band at the origin and was delivered from a 250 μl glass syringe through a stainless steel capillary needle (Scientific Glass Engineering Co. Ltd, Melbourne, Australia). The plates were developed for about 4 h in ascending n-butanol-acetic acid–water (4:1:5, v/v, upper phase) (Crammer, 1948) in a glass vessel in a light-tight incubator at 37°. The plates were then dried in a light-tight cabinet in a stream of hot air for 30–45 min. The bands fluorescing yellow in u.v. illumination were then isolated and eluted as already described. Mercury vapour lamps with Wood's glass filters (P. W. Allen and Co., London) were used as u.v. sources.

Need to exclude light

Solutions of isoalloxazines are photolabile (Owen & West, 1968*b*) and should be handled only in the dark. There is also evidence that midsummer sunshine at latitude 56° N can increase the riboflavine requirement of the white rat (Maslenikova, 1962). However, in each of two preliminary experiments with British Saanen female goats kept in a completely darkened room, there was no evidence that chromatograms of milk or urine were different from those obtained when the same animal was kept in daylight.

For the examination of chromatograms in u.v. illumination a darkened room is

essential. In urine especially, many blue-fluorescing substances can be seen, and sometimes substances with green or pink fluorescences were observed. Those substances with no yellow component in their fluorescence were not studied further. To guard against confusion due to artifacts we have subjected batches of chemicals of reagent grade used in extraction processes, e.g. riboflavine, trichloroacetic acid, phenol and others, to extractions and chromatography.

Materials

Riboflavine was bought either from the British Drug Houses Ltd, Poole, Dorset, or Koch-Light Laboratories, Colnbrook, Buckinghamshire.

FMF and HEF were prepared from riboflavine as described by Fall & Petering (1956) and were purified by preparative layer chromatography. To prevent adventitious contamination all glassware such as separatory funnels and burettes had polytetrafluoroethylene stopcocks.

Animals

British Saanen goats, Ayrshire cows and calves, sheep of various breeds and rabbits were used in these experiments.

Administration of riboflavine

Doses of riboflavine (1–5 g) were given orally, subcutaneously or by surgical fistula into rumen, abomasum or caecum. To prepare riboflavine for injection 1 g was suspended in a sterile solution of 50 g sucrose in 100 ml 0·9% (w/v) sodium chloride in water freshly distilled from glass so as to be pyrogen-free. This solution was further sterilized by immersion in a water-bath at 100° for 10 min. Half of it was injected into the right and half into the left flank of the animal, the total dose being less than one-sixteenth of the toxic limit of sucrose (Wattiaux, Wattiaux-de Coninck, Rutgeerts & Tulkens, 1964).

Expt 1

Eight British Saanen goats, four milking and four dry, were used to study the effect of riboflavine ingestion on the output of riboflavine and its metabolites in milk and urine. The diet of the animals was that used by Crossland *et al.* (1958), but chloride replaced sulphate in the salt mixture. The diet had the following percentage composition by weight: zein 17·4; potato starch 34·7; delignified straw 34·7; treacle 8·7; urea 0·5 and salt mixture 4. Every second day each goat was orally dosed with 50 mg α-tocopheryl acetate in 0·5 ml olive oil and 30 ml oil containing 2000 i.u. vitamin A and 200 i.u. vitamin D_2/ml (Crossland *et al.* 1958). The salt mixture (Miller, Price & Engel, 1956) had the following composition in parts by weight: $CaHPO_4$ 1000, $CaCO_3$ 360, NaCl 593, KCl 500, MgO 64, $FePO_4.4H_2O$ 37, $MnCl_2.4H_2O$ 21, KI 2·7, $ZnCO_3$ 1·8, $CuSO_4.5H_2O$ 1·2, potash alum 0·2, $CoSO_4$ 0·12.

On this diet riboflavine was the only isoalloxazine in the milk and urine of the goats unless riboflavine was fed to them. Since the diet was deficient in lysine, tryptophan and B-vitamins, the goats were dependent upon rumen bacteria for these nutrients.

Table 1. *Effect of an oral dose (100 mg) of riboflavine on the outputs of riboflavine* and total isoalloxazines in the milk and urine of goats which were on a diet† low in sulphate and deficient in B vitamins, lysine and tryptophan*

(All values are expressed as μg riboflavine (or equivalent isoalloxazine) per kg body-weight per day; each pre-dosing value is the mean of four, and each post-dosing value of two, 24 h collections of urine or milk. Goats nos. 1–4 were lactating; nos. 5–8 were dry)

		Urine			Milk				
		Before dosing	After dosing		Before dosing	After dosing			
Goat no.	Body-wt (kg)	Riboflavine	Total isoalloxazines	Riboflavine	Riboflavine	Total isoalloxazines	Riboflavine	% Dose recovered in milk + urine	Total isoalloxazines in rumen before dosing (ppm in dry matter)
1	53	39	91	60	37	46	46	3·2	13
2	48	35	87	37	56	71	N.D.	3·2	19
3	45	53	46	20	51	64	N.D.	0·3	21
4	45	31	83	53	40	71	63	3·7	25
5	41	10	31	12	—	—	—	0·9	11
6	61	21	117	32	—	—	—	5·6	37
7	45	35	79	36	—	—	—	2·0	10
8	61	29	45	14	—	—	—	1·0	11
Mean	49·9	31·6	72·4	33·0	46	63·0	51·0	2·5	18·4

* Riboflavine was determined microbiologically with *Lactobacillus casei* (ATCC 7469).

† The diet supplied about 7 μg riboflavine per kg body-weight per day.

The last column in Table 1 shows that, like the similarly treated goats of Crossland *et al.* (1958), the goats of Expt 1 did synthesize riboflavine in the rumen.

The effect of an oral dose of 100 mg riboflavine is shown in Table 1. Each goat was dosed twice and the values with each treatment were averaged to obtain the outputs shown in Table 1. The average output of riboflavine before the dosing of each goat, which was the average of four 24 h urine collections (Table 1) varied from 10 to 53 μg/kg body-weight per day. This variation was probably due mainly to variations in rumen synthesis. The diet itself provided only about 7 μg/kg body-weight per day. After the 100 mg dose of riboflavine the total isoalloxazines increased in every goat except no. 3. The contribution of true riboflavine to the total isoalloxazines was measured microbiologically, and the fact that this value was always considerably less than the value for total isoalloxazines measured fluorimetrically showed that every goat excreted in its urine some isoalloxazines as metabolic products of the administered riboflavine.

Comparison of the riboflavine outputs before and after dosing shows that the administered riboflavine did not significantly affect the output of this vitamin. Like others, we have repeatedly noted that very little of an oral dose of riboflavine reaches the urine or the milk. Table 1 shows that the recovery of riboflavine varied considerably from goat to goat but averaged only 2·5%, with 3·7% as the biggest value.

Expt 2

Eight milking goats, not given supplements of riboflavine, averaged 1224 ml urine per day for several days and excreted 41 μg riboflavine/kg body-weight per day. This output compares well with the urinary output of the four goats of Crossland *et al.* (1958), which was, on average, 69 μg riboflavine/kg body-weight per day. Another goat orally dosed with 1 g riboflavine excreted 986 ml urine on the following day, and this contained 64 μg riboflavine/kg body-weight per day together with metabolites that were equivalent, on the basis of fluorimetric measurements to four-and-a-half times the riboflavine.

Expt 3: fate of riboflavine in the caecum of goats

The caecum of a male goat was fistulated by the surgical technique described by Faitelberg & Semenjuk (1956), and when the wound had healed a suspension of 1 g riboflavine in artificial saliva (McDougall, 1948) was placed in the caecum at 11·45 h. By 09.15 h the following day HEF had not appeared in the urine.

Samples of caecal contents were withdrawn at various times and mixed with excess of trichloroacetic acid, ground in a mortar with sand, neutralized and tested by thin-layer chromatography. The chromatograms showed only riboflavine although incubation of the caecal contents for 2–3 days with riboflavine in vitro gave rise to demonstrable amounts of HEF (West & Owen, 1968).

An adult female goat with a caecal fistula was given 1 g riboflavine to eat with its food at 12.35 h and samples of the caecal contents were removed at hourly intervals thereafter. The extracts of the first four samples showed only blue-fluorescing mat-

erials, but at 5 and 6 h after the meal riboflavine was detectable in the caecum and at 7 h HEF accompanied riboflavine. By 9 h riboflavine, HEF and FMF were all found.

When 1 g riboflavine in 9 ml 0·2% agar was placed in the caecum of a fistulated milking goat, thin-layer chromatograms of milk samples taken for the following 22 h showed no metabolites. When, later, riboflavine was fed to this goat both milk and urine showed the expected metabolites.

These experiments show that metabolites produced in the rumen can escape absorption and so reach the caecum. They also show that conditions in the caecal contents in vivo are not conducive to the production of riboflavine metabolites in spite of the presence in the caecum of bacteria capable of producing HEF.

Expt 4: *time of appearance of metabolites after riboflavine ingestion*

An adult male goat was given 1 g riboflavine by mouth at 06.30 h. After 2 h riboflavine appeared on chromatograms of its urine and after 5 h a metabolite, RM3 (Owen, 1962*a*, *b*), appeared. After 6 h, another metabolite, RM2 (Owen, 1962*a*, *b*), also appeared but it was not until 16 h after the dosing that these excretory products were accompanied by HEF. Samples of urine voided during the next 13·5 h all showed each of these products, but after this lapse of time HEF had disappeared, and 20 h after the time of ingestion of the riboflavine the urine had returned to its pre-experimental state.

Expt 5: *effect of feeding to a kid the milk of a goat that had ingested riboflavine*

To test the ability of the young animal to excrete HEF and other metabolites in its urine when given those metabolites preformed, a milking goat was given riboflavine orally and the resulting yellow milk was collected for feeding to one of its twin kids (kid A). As can be seen from the chromatograms in Fig. 2 the dam's milk was richer than normal in riboflavine and, as expected, contained abundant HEF and other riboflavine metabolites. The concentrate from the yellow milk (Fig. 2) represented less milk than the pretreatment control and so showed no blue-fluorescing spots.

As a preliminary to this experiment kid A was given an oral dose of riboflavine (500 mg); paper chromatography (Crammer, 1948) showed that excess riboflavine unaccompanied by any metabolites then appeared in its urine. When daily chromatographic checks indicated that the kid's urine had returned to its pretreatment state, this kid was given the yellow milk of its dam. After 5 h, HEF and two other metabolites of R_F 0·2 and 0·18, were present on chromatograms. Blue-fluorescing material, of R_F 0·40, was also found but this was likewise present in the urine of the control kid B, collected at the same time. This type of chromatogram of kid A's urine persisted until 17.00 h on the following day, but after that time the chromatograms had reverted to the type with only three blue-fluorescing spots.

Thus, when goat's milk, containing HEF and other yellow fluorescing metabolites, is fed to a 14-day-old kid unable itself to produce those metabolites, HEF and other yellow fluorescing metabolites readily reach the urine of the kid.

Blue-fluorescing materials in urines 1, 4 and 7 which do not appear in 2, 5 and 8,

respectively, indicate that a greater amount of the former samples had to be used to produce their chromatograms, so that these blue-fluorescing materials were diluted away in 2, 5 and 8 (Fig. 2).

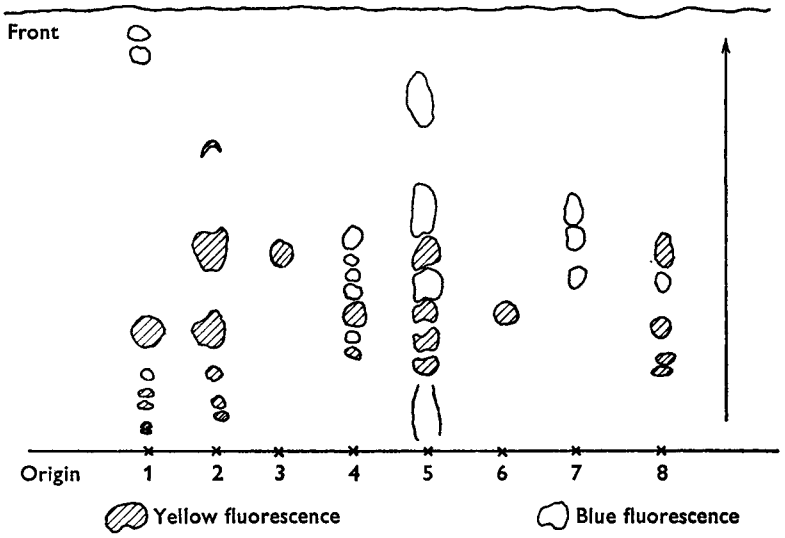

Fig. 2. Absorption and excretion of hydroxyethylflavine (HEF) as made evident by the transfer of HEF secreted in the milk of the mother goat to the urine of its kid (Expt 5). While the dam was producing HEF in both milk (chromatogram 2) and urine (chromatogram 5), its milk was fed to one of its kids the urine of which (chromatogram 8) shows HEF which was not present previously (chromatogram 7). The chromatograms were on paper and were developed by ascending solvent. The solvent was n-butanol–acetic acid–water (4:1:5; v/v; upper phase). (1) Extract of dam's milk before ingestion of riboflavine; (2) the same after ingestion of riboflavine; (3) HEF; (4) dam's urine before ingestion of riboflavine; (5) dam's urine after ingestion of riboflavine; (6) riboflavine; (7) kid's urine after it had drunk milk as in (1); (8) kid's urine after it had drunk milk as in (2).

Expt 6: *riboflavine given orally to kids*

Two male kids, C and D, one 16 and the other 17 days old, each received 1 g riboflavine in 1 pint of their dam's milk from a bottle at noon on one particular day. Urine samples taken before the administration of the riboflavine showed only four blue-fluorescing spots and one purple-fluorescing spot when chromatographed on paper in the Crammer (1948) system. No riboflavine was present. By 17.00 h, however, riboflavine was present on the chromatogram but was unaccompanied by HEF, the chief metabolite of adult animals. In the urine of both kids a yellow-fluorescent spot (X), of R_F about two-thirds that of riboflavine, appeared, and in addition the urine of kid D showed a second spot (Y), of R_F about half that of the riboflavine spot. However, by 17.00 h on the following day HEF had appeared on the chromatogram of the urine of kid C together with both spots (X) and (Y) and a fifth yellow-fluorescing material (Z) about midway between spot (X) and riboflavine. HEF was not apparent in the urine of kid D, but by noon the following day this metabolite was plainly visible on chromatograms of the urine of both the kids although it was much less distinct than it had previously been in the urine of kid C. At 14.00 h on that day riboflavine was still

plainly visible in the urine of kid C but HEF had disappeared, only to reappear in the urine of both kids at 09.00 h the following morning. At 14.00 h that same afternoon, i.e. 2 days after the administration of the riboflavine, each kid's urine showed riboflavine plainly, with faint indications of HEF and the other three yellow-fluorescing substances (X), (Y) and (Z). Of these last three substances (Z) was the most distinct. This type of chromatogram continued for 4 days after the time of administration of the riboflavine. By the 5th day, riboflavine had almost disappeared from the urine but a trace of HEF was just detectable. This experiment showed that, by about 2 weeks of age, the kid is beginning to develop the adult type of digestive process. Indeed this has long been known from other observations on the digestive processes of ruminants. However, the non-persistence of the excretion of riboflavine showed that rumination had not been properly established while the animals were still milk-fed. The intermittent appearance of the HEF can be explained by a later experiment on adult goats, in which the production of HEF in the rumen was studied concurrently with rumen pH, and it was found that HEF production was maximal when the pH of the rumen reverted to near neutrality after being lowered by the production of lower volatile fatty acids in the rumen immediately after feeding (West, Owen & Taylor, 1967).

Expt 7: fate of riboflavine injected subcutaneously into newborn kids

The two kids C and D of Expt 6 were used. Kid C was given, by subcutaneous injection, 0·84 g riboflavine in 50% (w/v) aqueous sucrose. There were no yellow-fluorescing materials in the chromatograms of the urine of the control animal D, but in the urine of kid C riboflavine appeared prominently and was unaccompanied by any other yellow-fluorescing substance. No metabolites could be detected. The urine of kid D showed, on ascending paper chromatograms in the Crammer (1948) solvent system, two purple- and seven blue-fluorescent spots, one of the latter being at the origin and one of the former almost at the front. In the chromatograms of the urine of kid C, all these spots were visible on u.v. illumination, but in addition there was the yellow spot in the riboflavine position. When blood samples from the kids were examined chromatographically, the blood of kid C showed a yellow-fluorescent spot near the origin in the position of the flavine–adenine–dinucleotide pilot spot. There was not enough of this material to be visible in the blood of kid D. The absence of metabolites from the urine of kid C implies that little of the injected riboflavine could have reached the rumen.

When 1 g riboflavine was eaten by a goat (Expt 4) the excretion of metabolites and additional riboflavine in urine and milk usually ceased after a few days, but when riboflavine was injected into kid C riboflavine excretion continued for a long time, and 22 days after injection the urine still showed a prominent yellow-fluorescing spot on chromatograms. At this time kid D was just beginning the excretion of the small amount of riboflavine in its urine that is a natural consequence of the establishment of rumination (Owen, 1954). At this time a quantitative experiment was carried out on kids C and D (Table 2). Kid D was given 1·8 g riboflavine by mouth. This caused metabolites HEF, RM 2 and RM 3 to appear in its urine and also increased the excretion of riboflavine. It can be seen that, within a day of receiving 1·8 g riboflavine by

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Table 2. *Expt 7. Effects of riboflavine, given orally or by subcutaneous injection, on its urinary excretion by kids*

Kid	Treatment with riboflavine	No. of days after dosing	Time of urine collection	Hydroxyethylflavine in urine	Volume of urine in single urination (ml)	Concentration of riboflavine in urine (ppm)	Output of riboflavine (μg/urination)
C	0·84 g (injection)	13	10.00	Absent	20	9·9	198
		14	18.30	Absent	21	27·9	620
D	1·8 g (orally)	0·5	11.45	Present	15	2·2	33
		1·0	21.20	Present	39	1·5	59

mouth, kid D had only 1·5 ppm of riboflavine in its urine, whereas kid C had 9·9 and 27·9 ppm in its urine 13 and 14 days respectively after the injection of only 0·84 g riboflavine. Whereas kid C's urinary excretion of riboflavine persisted for 22 days, the urine of kid D no longer showed yellow-fluorescing materials on chromatograms on the 7th day after it had had the much larger dose of riboflavine by mouth. Table 2 also shows that the concentration of riboflavine in the urine of the injected kid was much greater than the average concentration (1·8 ppm) found in the urine of four adult female milking goats that were eating conventional rations (Crossland *et al.* 1958).

Expt 8: *riboflavine given orally to pre-ruminant calves*

Urine samples were collected from two 5-day-old bull calves, A and B, and each was then given 0·5 g riboflavine in its milk at 16.00 h. Urine was collected from the calves after 5·75 h and 15·3 h after dosing. All four samples fluoresced yellow in u.v. light, whereas the pretreatment samples were blue-fluorescent. Thin-layer chromatography of concentrates prepared from the urine samples, with chloroform–methanol (9:1) as solvent, showed that the yellow fluorescence in the first samples taken after dosing was entirely due to riboflavine, but the second samples taken after dosing contained riboflavine and some HEF and FMF. Fourteen days after dosing, HEF could still be detected in the urine of these two calves.

Expt 9: *subcutaneous injection of riboflavine into the lactating goat*

Riboflavine (1·7 g in 50%, w/v, sucrose) was injected subcutaneously, half the dose into each flank, into a lactating female goat at 16.40 h. The following morning the milk was already yellow and was even yellower by evening. The urine, first examined on the 4th day, was strongly yellow-fluorescent in u.v. light. At this time the milk was still very yellow. Thin-layer chromatography in chloroform–methanol (9:1, v/v) revealed riboflavine in both milk and urine but no metabolites. After 6 days the colour of the milk and the yellow fluorescence of the urine still persisted, but both were due to riboflavine. Saliva, collected by the method of McDougall (1948), showed neither riboflavine nor its metabolites.

Seventeen days after injection this goat was still producing milk and urine containing excess riboflavine. It was not examined for another month, by which time both milk and urine had returned to normal.

Expt 10: *ingestion of FMF* (*Fig.* 1) *by the kid*

A male kid, 2 weeks old and weighing 9 kg, was given 160 mg of FMF (in the methanolate form) with its milk at 06.00 h. Thin-layer chromatography of urine showed abundant HEF and unchanged FMF in about equal amounts and some yellow-fluorescent material, which stayed at the origin in the chloroform–methanol (9:1) system. Evidently the aldehyde FMF is reduced in vivo to the alcohol HEF.

A young kid was then given 200 mg FMF with its milk, and its urine was found to contain more HEF than FMF, but both were present in quantities that were readily determined by thin-layer chromatography. Both HEF and FMF were also found when

bile and caecal contents were examined *post mortem*, but no HEF was found in the rumen. The bile resembled the urine in containing more HEF than FMF, but in the caecum FMF was preponderant.

Expt 11: *injection of FMF into kids*

At 09.00 h, 1·2 mg FMF was injected subcutaneously into a male kid. By 11.30 h the urine was yellow and thin-layer chromatography showed both FMF and HEF in the proportions of about 1·5 to 1. Both these substances were still visible by thin-layer chromatography at 12.20 and 13.10 h. This finding showed that the reduction of FMF to HEF can occur in the tissues of the kid (See Expt 15).

Expt 12: *fate of riboflavine placed in the abomasum of the sheep by means of a fistula*

An adult sheep with a fistula into the abomasum was given in the morning 1 g of riboflavine in 0·2% agar directly into the abomasum. Urine collected before dosing showed, on thin-layer chromatography with the Crammer (1948) solvent system, riboflavine as the only yellow-fluorescent material, though blue- and purple-fluorescent materials were also visible. The same afternoon at 16.00 h and at 17.30 h the urine still showed riboflavine as the only fluorescent material and the pattern was the same at 09.00 h the following day. Such a dose of riboflavine would have caused a large output of metabolites had it been given by mouth or placed in the rumen. Similar results were obtained with a second sheep. Thus riboflavine entering the gut distally to the rumen did not cause the appearance of urinary metabolites.

Expt 13: *natural occurrence of hydroxyethylflavine* (*HEF*)

With the improvement of our methods of extraction and detection of HEF and FMF it became possible to see whether these isoalloxazines occur naturally. The first animal to be studied was the rabbit, which, being entirely herbivorous and having a caecum that is the largest organ in its body, is independent of an external source of vitamins of the B-complex. Urine from rabbits fed on grass and weeds was found to contain HEF, but analysis failed to show HEF in either of these foods. We therefore concluded that HEF occurs naturally in the rabbit.

We also found, by concentrating solvent extracts of urine and rumen contents, that, on normal diets the rumen of the cow and the urine of the sheep and goat contain HEF.

Expt 14: *post-mortem examination of animals dosed with riboflavine*

(*a*) After having eaten 3 g of riboflavine on the previous day, a weaned male kid was killed. At autopsy HEF could not be found in diaphragm, rumen wall, caecal wall, omental fat, liver, abomasal wall, reticulum wall, kidney, pancreas, brain, testis, heart or spleen, but was found in rumen contents, faeces, colon contents, caecal contents, rectal contents and in the contents of the small intestine. The chromatogram prepared from lung tissue showed a long continuous yellow-fluorescing streak in which discrete spots could not be distinguished. Of considerable interest was the finding of

HEF in bile taken from the gall-bladder. Similar observations were made on a second male kid which had ingested 2 g riboflavine and on an adult female goat which had eaten 1 g riboflavine. In both these animals HEF was found in bile.

(*b*) A sheep, killed after 1 g riboflavine had been administered through an abomasal fistula, was examined *post mortem*. No HEF was found in the wall or contents of the ileum, caecum or colon, or in the contents of the abomasum, bladder or gall-bladder. This absence of HEF in the bile contrasts with its presence in the animals in (*a*).

(*c*) Flavine-adenine mononucleotide (riboflavine-5′-phosphate) was fed to a 10-day-old lamb, 2 h before it was killed. Free riboflavine, unaccompanied by metabolites, was found in urine and bile.

Expt 15: *metabolism of riboflavine in tissues in vitro*

Beef liver and kidney were obtained fresh from Ayr slaughterhouse, and the same tissues were taken from surplus male kids from the Biochemistry Department's herd of goats. These tissues were incubated in Ringer–Locke solution, with or without riboflavine, at blood heat (40°) and flavines were extracted with trichloroacetic acid (Bessey *et al.* 1949). Paper chromatograms (Crammer, 1948) showed riboflavine, whether or not it had been added to the medium, thus confirming the previous observations of Owen & Dzialoszynski (1965*a*). This riboflavine would arise from the autolysis of flavine-adenine dinucleotide and flavine mononucleotide, which are known to be relatively abundant in these tissues. There were, however, no yellow-fluorescing substances of R_F greater than that of riboflavine (0·28), so that neither HEF nor FMF was produced.

When either liver or kidney was incubated with FMF part of the FMF was reduced to HEF.

CONCLUSIONS AND DISCUSSION

The foregoing experiments show that metabolites of riboflavine appear in urine, milk and bile when riboflavine is given by mouth, but not when it is administered by subcutaneous injection or by means of a fistula into a part of the gut that is distal to the rumen. Oral administration of riboflavine is followed by the appearance of its metabolites in the rumen. Since rumen contents incubated in vitro produce metabolites it can be concluded that the rumen is the site of production of the metabolites of riboflavine in milk, urine and bile.

After oral riboflavine, metabolites were found in the caecum but not in the urine, and, when caecal contents were incubated in vitro with riboflavine, metabolites were formed. Evidently the metabolites are not absorbed from the ruminant's hind-gut.

When riboflavine is injected subcutaneously it reaches the milk, the bile and the urine but is unaccompanied by any of the metabolites.

In accord with what we may call the symbiotic hypothesis that the rumen is the site of production of metabolites of riboflavine, we found that neither the liver nor the kidney is able to produce them in vitro from riboflavine. These tissues can, however, reduce FMF to HEF in vitro, and this action probably explains our observations that subcutaneously injected FMF is partly excreted in the urine as HEF. In confirmation

of the symbiotic hypothesis, we have recently isolated from rumen contents species of bacteria that give rise, when grown in isolation in vitro, to riboflavine metabolites. Pure cultures of such bacterial species are being studied now (Hobson *et al.* 1969).

The symbiotic hypothesis also explains the absence of metabolites of ingested riboflavine from the urine of the dog (Owen & Dzialoszynski, 1965*b*) and the normal presence of HEF in the urine of the rabbit. It likewise explains the absence of metabolites when riboflavine is fed to newborn kids in spite of their ability to excrete HEF given to them in their dam's milk. Consistent with the hypothesis also was our failure to find metabolites in the ruminant's tissues, although we found them in the gut contents, the bile, the urine and the milk.

The non-absorption of metabolites from parts of the gut distal to the rumen presents an analogy with the relation of cobalt to pining in lambs. Administration of Co increases the production of vitamin B_{12} by rumen bacteria and so is able to cure the disease. If the Co salts are injected they give rise (see review by Owen, 1959) to vitamin B_{12} in the hind gut but do not cure the disease. The rabbit, because of its habitual coprophagy, overcomes this difficulty by subjecting a portion of its food to a repetition of the whole process of digestion, so presenting the HEF formed in the caecum to the absorptive parts of its gut and so to its tissues and urine (Owen, West & Coates, 1970).

Expt 8 gave a result requiring comment. It records the occurrence of HEF and FMF in the urine of calves only 7 days old. In this experiment concentrated extracts of the urines were used for thin-layer chromatography instead of the urine itself so that traces of HEF and FMF that might normally have escaped detection were found. There may also be an oecological reason for this observation, for the time of onset of fermentation in the rumen is arbitrary, depending as it does on the nature and intensity of the initial contamination of the calf's food with the bacteria and protozoa that find in the rumen their preferred habitat.

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