The urinary excretion of metabolites of riboflavine by man

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1. A dose of 1 g of riboflavine caused a large excretion of the vitamin in human urine and the rate of excretion showed more than one maximum.

2. Various degradation products of riboflavine appeared in the urine approximately 24 h after the dose was administered and two of these were identified as 7,8-dimethyl-10-(2-hydroxyethyl)isoalloxazine, and 7,8-dimethyl-10-formyl-methylisoalloxazine. Reasons are given for believing that the degradation of riboflavine was due to bacterial action in the colon.

The riboflavine requirements of different species of animals under various nutritional and physiological conditions have been studied by many investigators and their results have been reviewed (Bro-Rasmussen, 1958*a*, *b*). Although the enzymic functions of and nutritional requirements for this vitamin have been well established the need for the continual ingestion of riboflavine by adult animals is not so well understood. Bessey, Lowry, Davis & Dorn (1958) attributed this 'maintenance requirement' of riboflavine to decomposition of the vitamin by the tissues, but Yang & McCormick (1967), using [^{14}C]riboflavine at a level equivalent to the normal physiological dose of the vitamin, showed that the bulk of the administered riboflavine was excreted in the urine. At most, 25 % could have been destroyed and they attributed this destruction to the action of intestinal bacteria rather than of the tissues themselves. The ability of intestinal bacteria to decompose riboflavine was demonstrated by Teshima & Kashiwada (1966), who worked with bacteria obtained from the carp, and by Innami, Kawachi & Oizumi (1965), who reported various yellow-fluorescing, chloroform-soluble metabolic products in the faeces of rats maintained on a diet rich in riboflavine. Similar metabolic products were also found in rat faeces by Yang & McCormick (1967), who however did not report any such degradative products in the urine of their animals. In addition we have shown (West, Owen & Taylor, 1967) that the rumen and caecum of goats harbour organisms that are capable of degrading riboflavine to yellow-fluorescing products among which are 7,8-dimethyl-10-(2′-hydroxyethyl)-isoalloxazine (hydroxyethylflavine) and 7,8-dimethyl-10-formylmethylflavine-isoalloxazine (formylmethylflavine).

In view of the results of Yang & McCormick (1967), and particularly in view of the results of our own studies of caecal bacteria in vitro, we wished to find out whether human beings would excrete any degradative products of riboflavine after ingestion of large doses of the vitamin. Najjar, Johns, Medairy, Fleischmann & Holt (1944) had shown that colonic synthesis and absorption of riboflavine can occur in man and it therefore seemed reasonable to suppose that any degradation products resulting from the activity of intestinal bacteria would be similarly absorbed and excreted.

Several groups of workers (e.g. Stripp, 1965; Jusko & Levy, 1966; Spencer &

Zamcheck, 1961) have studied the absorption of riboflavine in man with a view to determining the mechanism of absorption of the vitamin, but only Owen & Dzialoszynski (1965) made any attempt to look for metabolic products of the vitamin in human urine.

It is the purpose of the present paper to show that metabolic products of riboflavine are excreted in human urine and to show that two of these products, hydroxyethylflavine and formylmethylflavine, are identical to the metabolites isolated from ruminant urine (Owen & West, 1970).

MATERIALS AND METHODS

Materials. Riboflavine was purchased from Koch–Light Laboratories (Colnbrook, Bucks.), Woelm neutral alumina for column chromatography from Camlab (Glass) Ltd (Cambridge), and Camag Kieselgel D5 for thin-layer chromatography from Griffin & George (East Kilbride, Glasgow). 7,8-Dimethyl-10-(2′-hydroxyethyl) isoalloxazine, its acetyl derivative and 7,8-dimethyl-10-formylmethyl-isoalloxazine, were prepared according to the method of Fall & Petering (1956). Solvents for chromatography were May & Baker 'R' grade and were used without further purification.

Thin-layer chromatography. Silica gel thin-layer chromatoplates of 250 μm thickness were used with the following four solvent systems: (*a*) chloroform–methanol (9:1, v/v), (*b*) butanol–ethanol–water (7:2:1, v/v), (*c*) benzene–glacial acetic acid–pyridine (4:1:1, v/v) and (*d*) butanol–glacial acetic acid–water (4:1:5, v/v, upper phase). System (*a*) gave the most rapid development and was used for all the quantitative chromatography. The other three systems were mostly used for the comparison of the R_F values of the isolated compounds with authentic materials. On these occasions the unknown material was applied as a band across a portion of the bottom of the plate and both internal and external marker spots of the authentic materials were used. The isoalloxazines were located after chromatography by their fluorescence in long wave-length u.v. light.

Analytical methods. Riboflavine and its metabolites present in the urine were determined fluorimetrically with a Locarte Mk IV fluorimeter with primary filter LF.3 and secondary filter LF.7. A modified procedure (US Pharmacopoeia, XVI, 1960) was used to assay the control urines. In this procedure the urine (5 ml) was treated with 1 M-acetate buffer (pH 4·8, 1 ml), 1 % (w/v) potassium permanganate (1 ml) and 3 % (v/v) hydrogen peroxide (1 ml) in that order and then made up to 10 ml in a volumetric flask. The fluorescent intensity of this solution was then measured in the fluorimeter, and the non-specific fluorescence was measured after reduction of the isoalloxazine with solid sodium hydrosulphite. Before addition of the sodium hydrosulphite, solid sodium carbonate was added to bring the solution to neutrality and thus to prevent the formation of colloidal sulphur. The amount of isoalloxazine present was then calculated by reference to a calibration curve prepared by measuring the fluorescence of various dilutions of a standard riboflavine solution (5 mg/l).

The presence of riboflavine and its degradative products in the urines was

investigated initially on small (10 × 15 cm) thin-layer chromatoplates developed in solvent system (*a*). In this way the presence and position of each yellow-fluorescing metabolite was established so that in the quantitative assay, using preparative layer chromatoplates, no internal markers were required.

For the measurement of the amount of riboflavine and its metabolites present in the urine, silica gel preparative layer chromatography was used, the urine under investigation (200 μl) being applied to the silica as a band across the bottom of the chromatoplate. Each plate was repeatedly developed in the solvent system (*a*) until a satisfactory resolution had been obtained. In this solvent system riboflavine hardly moves from the origin but the main metabolite (hydroxyethylflavine) separates well on repeated development. The plates were dried in a current of warm air, and each yellow-fluorescing band on the silica was located, scraped off the plate and the fluorescent material was eluted with water (5 × 2 ml) by the method of Owen (1968). The fluorescence of these solutions was measured against that of a standard riboflavine solution, as stated above Although flavines have the same fluorescent intensity on a molar basis as riboflavine, the recovery of each of the metabolites of known structure was estimated using the appropriate molecular weight. This is not valid for the unidentified metabolite 'A' which is referred to later, and the values for that material were obtained on the assumption that it had the same molecular weight and fluorescent intensity as riboflavine and no account was taken of possible internal quenching such as occurs with FAD.

Since the US Pharmacopoeia assay depends only on fluorescence each control urine was tested qualitatively on thin-layer plates. As an additional check, one of the control urines was assayed by both procedures. Fluorimetrically it gave 1·54 and chromatographically 1·48 mg/24 h. When riboflavine was added to control urine 95% was recovered from thin-layer chromatograms and results were corrected accordingly.

The percentage of ingested riboflavine recovered from each subject was calculated after deduction of the amount of riboflavine (see Table 1) found in his control sample of urine.

To avoid photolytic decomposition of the isoalloxazines all analyses were carried out in a dark room

Collection of urine samples. Urine samples were provided by five healthy male volunteers ranging in age from 28 to 60 years. Each subject received 1 g riboflavine suspended in a small amount of treacle approximately 1·5 h after breakfast, except subject W, who received a dose 1 h after the midday meal. No effort was made to keep the subjects on a restricted diet or to instruct them to increase their intake of fluid. Each urination was collected *in toto* in an amber glass bottle and was investigated immediately or, when this was impracticable, chloroform (1 ml/100 ml urine) was added and the bottles were placed in a refrigerator. The delay between collection and analyses was never more than 24 h.

From each subject a control sample of urine was collected during the 24 h period immediately preceding the ingestion of the dose of riboflavine.

RESULTS

During the first 20–24 h riboflavine, unaccompanied by any other isoalloxazine, occurred in the urines of all five subjects. However, the thin-layer chromatograms of the urines collected during the second 24 h after dosing clearly showed, in addition to riboflavine, a second band of yellow-fluorescing material which was identified as hydroxyethyl-flavine and a third band of yellow-fluorescing material, substance 'A' of Table 2. The R_F value of substance 'A' was just greater than the R_F value of riboflavine. Partly because of the spread of the more prominent riboflavine band and partly because of the presence of varying amounts of contaminating salts in the urines, compound 'A' was not always well resolved from riboflavine. For subject D the resolution was particularly troublesome, and although there were indications that compound 'A' was present in his urine samples, it proved impossible to obtain a sufficiently good resolution of it from riboflavine. In this instance the two bands were removed from the plates together and were treated as one band for the measurements of fluorescence.

Finally, in four of the urine samples of subject W a fourth yellow-fluorescing band was observed. This had an R_F value in chromatographic system (*a*) that was the same as formylmethylflavine, which has recently been shown to be a product of the bacterial degradation of riboflavine (Owen & West, 1968).

Identification of the metabolites, hydroxyethylflavine and formylmethylflavine. The urines containing the largest amounts of the main metabolite were combined, acidified to pH 4 with concentrated sulphuric acid and extracted with chloroform in a continuous extractor for 48 h. The chloroform extract was dried over anhydrous sodium sulphate and concentrated to small bulk on a rotatory evaporator at a temperature below 40°. On thin-layer chromatography this concentrate was found to consist of several blue-fluorescing materials, and one yellow-fluorescing compound which had the same R_F value as an authentic sample of hydroxyethylflavine in all four solvent systems. The chloroform concentrate was painted on to silica gel preparative layer chromatoplates which were developed in solvent system (*a*). The silica containing the yellow-fluorescing band was scraped from the plates and extracted with pyridine. Attempts to crystallize the material from the pyridine having been unsuccessful, the dry pyridine solution was mixed with freshly distilled acetic anhydride and the solution left at 37° overnight. After 24 h, by which time thin-layer chromatography indicated that the compound had been completely converted into the acetyl derivative, methanol was added to react with excess acetic anhydride and the solution evaporated to dryness. The residue was redissolved in chloroform and added to the top of a 28 × 1 cm column of neutral alumina, which had been prepared from a chloroform slurry. The column was washed with several volumes of chloroform, after which 2% (v/v) ethanol in chloroform eluted the desired material. The acetyl derivative of the metabolite crystallized out when the eluate was left to stand. It had the same R_F as an authentic sample of 7,8-dimethyl-10-(2′-acetoxyethyl) isoalloxazine in the four solvent systems and this, together with its infrared spectrum, confirmed that the metabolite was in fact hydroxyethylflavine.

The fourth metabolite (formylmethylflavine) was obtained by a continuous chloroform extraction of the appropriate four acidified urine samples of subject W and was separated from the contaminating hydroxyethylflavine by preparative layer chromatography in solvent system (*a*). This material would not crystallize from chloroform but it had the following properties in common with authentic formylmethylflavine: (1) it possessed the same R_F value as the authentic material in the four solvent systems, (2) it was almost quantitatively reduced by sodium borohydride to a flavine which corresponded in R_F value to hydroxyethylflavine, (3) upon treatment in the dark with 2 N-NaOH it was converted into a compound corresponding in R_F value to lumiflavine and (4) its u.v. absorption spectrum varied with pH in a manner similar to that shown by authentic formylmethylflavine (Smith & Metzler, 1963).

Investigations to establish the identity of compound 'A' are in progress.

Excretion of riboflavine. Table 1 shows, for each of the five subjects, the amount of riboflavine ingested and the amount of riboflavine excreted in the urine on each of the two following days, each urinary excretion having had the subject's normal basal excretion deducted. This basal excretion is recorded in the last column of Table 1. The total excess excretion varied from 63·7 mg for subject W to only 1·6 mg for subject T and averaged 30·9 mg, which was only 3·1 % of the dose. The bulk of the excretion occurred in the first 24 h after dosing and the recovery of the excess vitamin in the second 24 h period varied from zero for subject T to 25 % for subject D. These results are in agreement with the observations of others, notably Everson, Wheeler, Walker & Caulfield (1948), whose subjects also exhibited wide differences in the amount of riboflavine that was excreted in the urine. Subject T, who excreted very little of the administered riboflavine in his urine, was given a second 1 g dose of the vitamin to ascertain whether this low output was due to a poor nutritional status with regard to riboflavine or whether it was characteristic of this subject. His excretion of only 4·5 mg of this second dose indicated that his previous low output was characteristic.

Table 1. *Riboflavine in urine of human subjects after its oral administration*

		Riboflavine in urine in excess of basal excretion				
		Amount (mg)			Percentage of dose of riboflavine recovered in 2 days	Basal excretion: riboflavine in the 24 h control urine (mg/day)
Subject	Dose (mg)	1st day	2nd day	Total		
D	993	15·1	5·2	20·3	2·0	1·12
E	993	32·4	0·5	32·9	3·3	0·97
J	982	32·7	3·5	36·2	3·7	0·93
T	992	1·6	0·0	1·6	0·2	1·54
W	981	58·5	5·2	63·7	6·5	1·05
(Averages)	988	28·1	2·9	30·9	3·1	1·12

Fig. 1 shows histograms, plotted on a semi-logarithmic scale, of the variation of the rate of excretion of riboflavine throughout the period of the experiment. As can be seen, the riboflavine was rapidly excreted into the urine, with the maximum rate of excretion occurring within a few hours of dosing. The rate of excretion then declined

over the next 16–20 h, but during the second 24 h of the experiment the excretion rate of each subject increased and then declined to the pretreatment level. This second peak in the urinary excretion rate was small in comparison with the original one but was nevertheless quite distinct.

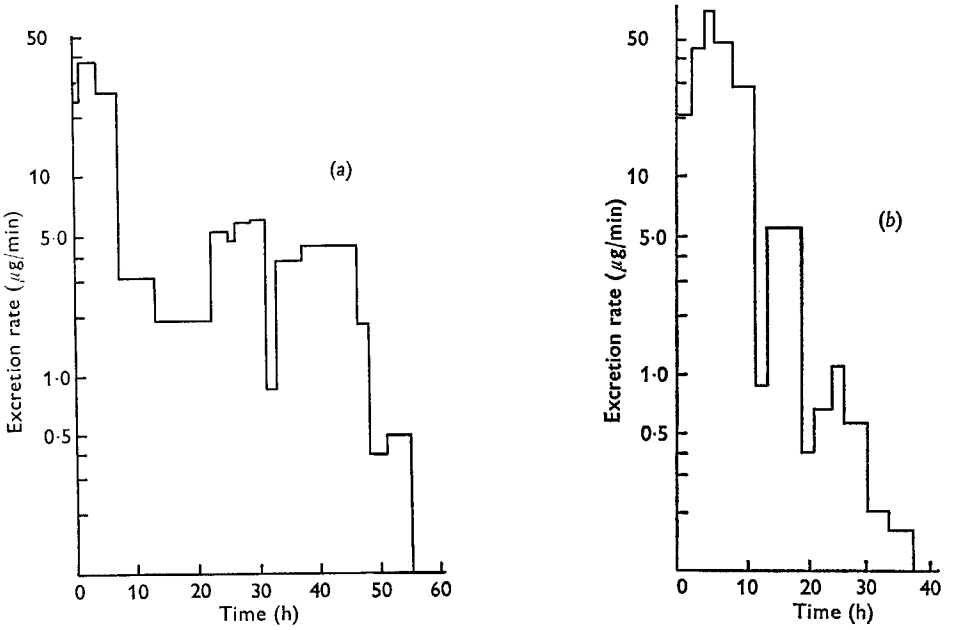

Fig. 1. Excretion of riboflavine in human urine: excretion rate as a function of time from ingestion of 1 g riboflavine (basal excretion of riboflavine not deducted). (*a*) Subject D, (*b*) subject E, (*c*) subject J, (*d*) subject T, (*e*) subject W. For *c*, *d* and *e* see opposite.

Excretion of metabolites of riboflavine. The total output of hydroxyethylflavine for each of the five subjects averaged less than 0·3 % (range 0·04–0·62) of the administered dose of riboflavine (Table 2). Hydroxyethylflavine first appeared in the urine samples about a day after the ingestion of the riboflavine, and its appearance generally coincided with the secondary increase in the rate of excretion of riboflavine. This metabolite occurred at times, and in amounts, that varied from subject to subject (Table 2); its pattern of excretion for each subject varied more than did that of riboflavine. In the two subjects, D and W, who had the highest and most protracted output of it, hydroxyethylflavine appeared in twelve separate urinations (Fig. 2), whereas the other three subjects excreted the metabolite in only five urinations, and their total output was much smaller. The hydroxyethylflavine excretion of subjects D and W exhibited several maxima (Fig. 2) and continued for many hours after the amount of riboflavine in the urine had reached pretreatment levels.

Compound 'A' was measurable in the urines of only three of the subjects (Table 2) and like hydroxyethylflavine it appeared in the urine after a delay of over 24 h. It was present to a much smaller extent than hydroxyethylflavine and never amounted to more than 0·05 % of the dose of riboflavine although, as mentioned earlier, no account is taken of any possible internal quenching when calculating this figure.

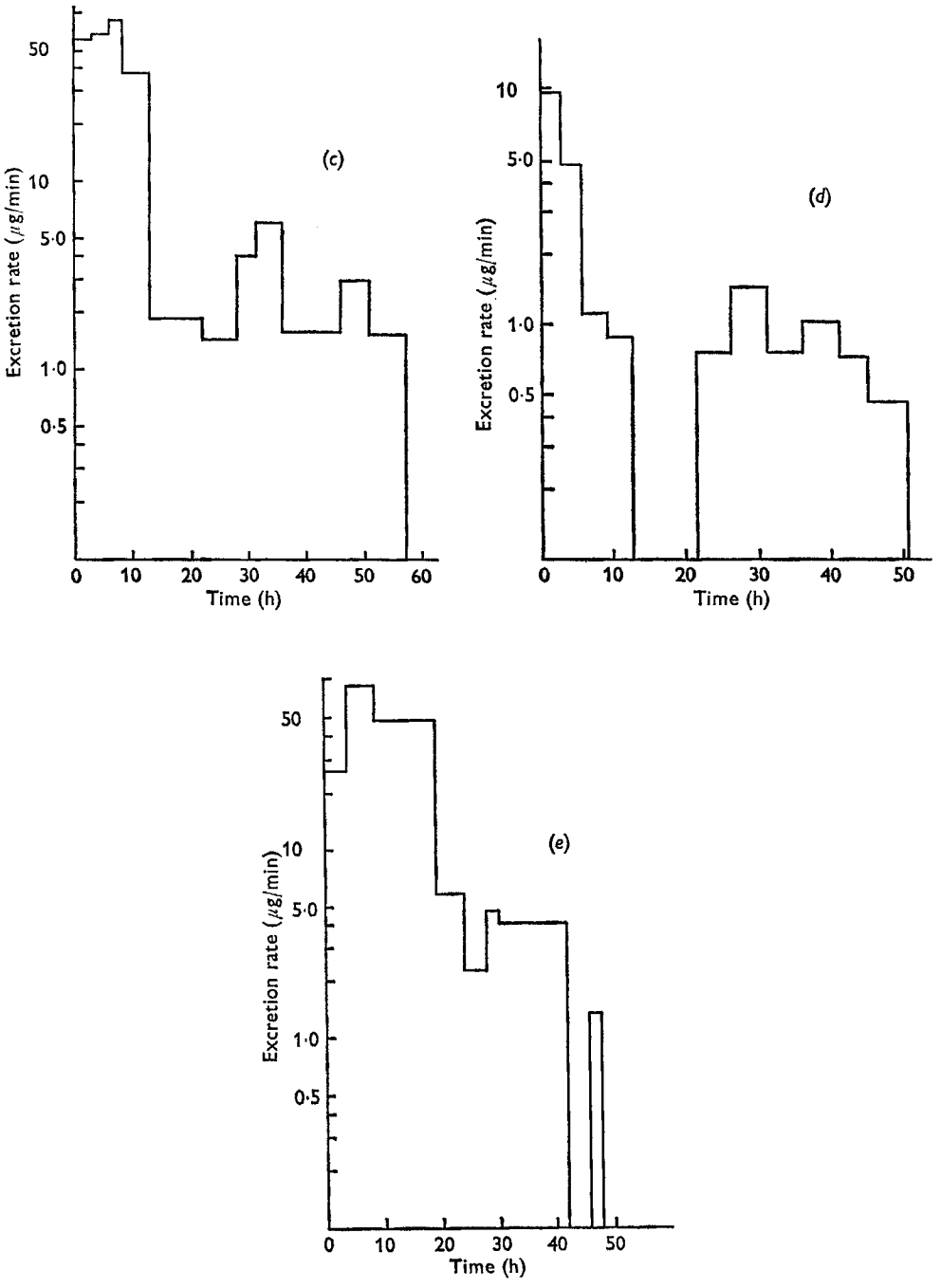

Fig. 1 *c*, *d* and *e*. For legend see opposite.

Formylmethylflavine was detected in the urine obtained from subject W to the extent of 0·03 % of the administered riboflavine (Table 2). This subject excreted the largest amount of both hydroxyethylflavine and riboflavine and his intestinal tract was obviously well adapted to the absorption of flavines. This may account for the occurrence of this material in his urine but not in that of any of the others.

Table 2. *Quantities of metabolites of riboflavine in human urine after oral administration of riboflavine, and the time that elapsed from ingestion of the vitamin to their first appearance in the urine*

	Hydroxyethylflavine			Compound 'A'			Formylmethylflavine		
Subject	Time (h)	Amount (g)	Percentage of dose	Time (h)	Amount (g)	Percentage of dose	Time (h)	Amount (g)	Percentage of dose
D	22·0	1498	0·15	—	0·0	0·0	—	0·0	0·0
E	27·0	371	0·04	—	0·0	0·0	—	0·0	0·0
J	31·5	742	0·08	46·0	383	0·04	—	0·0	0·0
T	26·0	801	0·08	26·0	511	0·05	—	0·0	0·0
W	23·0	6071	0·62	30·0	72	0·01	23·0	339	0·03

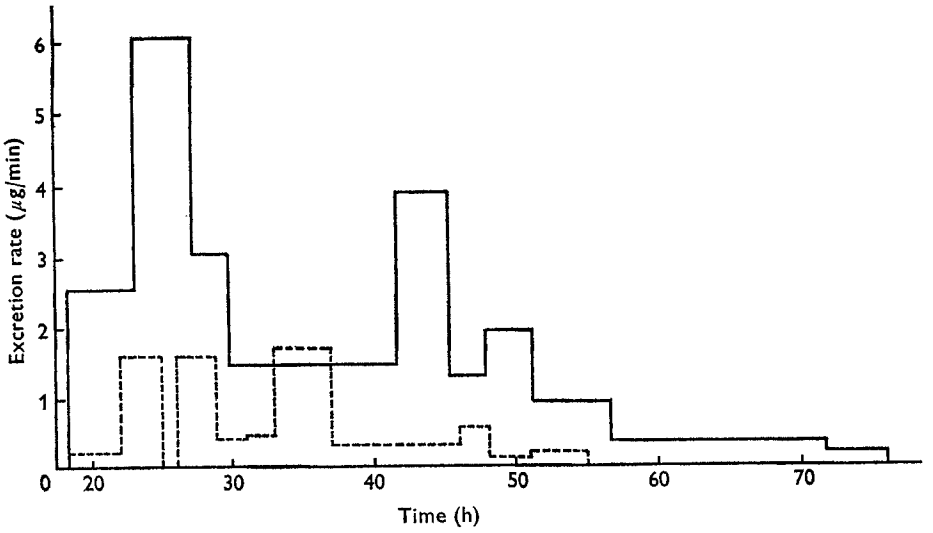

Fig. 2. Excretion of hydroxyethylflavine in human urine: excretion rate as a function of time from ingestion of 1 g riboflavine. ——, subject W; - - - -, subject D.

DISCUSSION

Formation of riboflavine metabolites. The results of the present experiments show that riboflavine is decomposed within the human body to various products such as hydroxyethylflavine in which the isoalloxazine nucleus is still intact. In these experiments we did not look for products in which the isoalloxazine nucleus was disrupted, although Yang & McCormick (1967) thought that such decompositions occurred to a small extent in rats.

When Owen & Dzialoszynski (1965) incubated riboflavine with slices of liver and kidney of ruminants no degradation products of the vitamin could be detected, a result that is in accord with similar experiments performed by Yang & McCormick (1967) on many different body tissues of the rat. In addition, Yagi, Nagatsu, Nagatsu-Ishibashi & Ohashi (1966), measuring the rate of migration of [^{14}C]riboflavine injected into rats, have reported that riboflavine is not decomposed in the mammalian body, and Owen & West (1970) showed that, although ruminants invariably excrete metabolites in their urine after eating riboflavine, administration of the vitamin by subcutaneous injection produces only riboflavine in the urine. To our knowledge there is no evidence that animal tissues can degrade riboflavine.

It is, however, well established that many bacteria are capable of degrading riboflavine both anaerobically and aerobically (Yanagita & Foster, 1956; Harkness, Tsai & Stadtman, 1964). In particular, Miles & Stadtman (1955) have reported an anaerobic soil organism which is capable of producing hydroxyethylflavine from riboflavine, and West *et al.* (1967) have found anaerobic organisms in the rumen and caecum of cows and goats which can produce both hydroxyethylflavine and formylmethylflavine from riboflavine.

It is therefore probable that the metabolites of riboflavine which appear in human urine are due to the activity of normal colonic bacteria and not to any degradative ability of the body tissues.

The induction of microbial enzyme activity by an enriched medium is a well-known phenomenon and the limited absorption of riboflavine in the small intestine (Jusko & Levy, 1966; Stripp, 1965) would ensure that the bacteria in the hind gut would be presented with an unusually large amount of the vitamin. The 24 h delay of the appearance of the metabolites in the urine would therefore allow sufficient time for the riboflavine to stimulate the activity of any riboflavine-degrading organisms present in the large intestine so that metabolic products could be produced in quantities sufficient for their detection in the urine.

Absorption and excretion of riboflavine and its metabolites. Several groups of workers (Jusko & Levy, 1966, 1967*a*,*b*; Stripp, 1965; Campbell & Morrison, 1963; Everson *et al.* 1948) have shown that for small doses of riboflavine there is a linear relationship between the amount of the vitamin administered orally and the amount recovered in the urine. Nevertheless there does appear to be a limit to the amount of the vitamin that can be absorbed, for both Stripp (1965) and Jusko & Levy (1967*a*) showed that with increasing amounts of riboflavine a decreasing percentage of the dose is recoverable in the urine. Thus Stripp (1965) recovered 17·9 mg of riboflavine after oral administration of 500 mg whilst Jusko & Levy (1967*a*), using the more soluble riboflavine-5′-phosphate, were able to recover 25 mg from a 300 mg dose.

In the present experiments in which 1 g riboflavine was fed to each of five subjects we have extended the observations to doses of riboflavine larger than those previously reported, but have observed that essentially the same average amount was recovered over the 1st day. However neither Stripp (1965) nor Jusko & Levy (1967*a*) continued collection for more than 24 h and we have been able to show that during the second 24 h after dosing a second peak of riboflavine excretion occurs and that this second

peak occurs at about the same time as metabolites appear in the urine. In addition if urine collection is continued for the 3rd and 4th day a good proportion of the fluorescence of the urine is due to the presence of the metabolites.

The reason for the second peak of riboflavine excretion is unknown at present. The fact that it occurs at the same time as metabolites appear in the urine suggests that it may be due to colonic absorption, although colonic absorption of riboflavine has been shown to be small (Jusko & Levy, 1966; Kuvaeva, 1967).

There is also the possibility that the insolubility of riboflavine may cause it to lodge on the gut lining, to be dislodged and absorbed at a later time. Further research is needed to determine the relative importance of these possibilities.

The attribution of the formation of the metabolites to the action of colonic bacteria can also readily explain the variation in the output of these compounds since pH, redox potential and other conditions of bacterial growth in the colon will vary both from subject to subject and with time of day in each subject.

REFERENCES

Bessey, O. A., Lowry, O. H., Davis, E. B. & Dorn, J. L. (1958). *J. Nutr.* **64**, 185.
Bro-Rasmussen, F. (1958*a*). *Nutr. Abstr. Rev.* **28**, 1.
Bro-Rasmussen, F. (1958*b*). *Nutr. Abstr. Rev.* **28**, 369.
Campbell, J. A. & Morrison, A. B. (1963). *Am. J. clin. Nutr.* **12**, 162.
Everson, G., Wheeler, E., Walker, H. & Caulfield, W. J. (1948). *J. Nutr.* **35**, 209.
Fall, H. H. & Petering, H. G. (1956). *J. Am. chem. Soc.* **78**, 377.
Harkness, D. R., Tsai, L. & Stadtman, E. R. (1964). *Archs Biochem. Biophys.* **108**, 323.
Innami, S., Kawachi, T. & Oizumi, H. (1965). *Jap. J. Nutr.* **23**, 1.
Jusko, W. J. & Levy, G. (1966). *J. pharm. Sci.* **55**, 285.
Jusko, W. J. & Levy, G. (1967*a*). *J. pharm. Sci.* **56**, 58.
Jusko, W. J. & Levy, G. (1967*b*). *J. pharm. Sci.* **56**, 1145.
Kuvaeva, I. B. (1967). *Fiziol. Zh. SSSR* **53**, 835.
Miles, H. T. & Stadtman, E. R. (1955). *J. Am. chem. Soc.* **77**, 5746.
Najjar, V. A., Johns, G. A., Medairy, G. C., Fleischmann, G. & Holt, L. E. (1944) *J. Am. med. Ass.* **126**, 357.
Owen, E. C. (1968). *Lab. Pract.* **17**, 1137.
Owen, E. C. & Dzialoszynski, L. (1965). *Proc. Nutr. Soc.* **24**, ix.
Owen, E. C. & West, D. W. (1968). *J. chem. Soc.* (C) p. 34.
Owen, E. C. & West, D. W. (1970). *Br. J. Nutr.* **24**. (In the Press.)
Smith, E. C. & Metzler, D. E. (1963). *J. Am. chem. Soc.* **85**, 3285.
Spencer, R. P. & Zamcheck, N. (1961). *Gastroenterology* **40**, 794.
Stripp, B. (1965). *Acta pharmac. tox.* **22**, 353.
Teshima, S. & Kashiwada, K. (1966). *Kagoshima Daigaku Suisan Gakabu Kiyo* **15**, 1.
US Pharmacopoeia, XVI (1960). 16th Revise, p. 907.
West, D. W., Owen, E. C. & Taylor, M. M. (1967). *Proc. Nutr. Soc.* **26**, xvii.
Yagi, K., Nagatsu, T., Nagatsu-Ishibashi, I. & Ohashi, A. (1966). *J. Biochem., Tokyo* **59**, 313.
Yanagita, T. & Foster, J. W. (1956). *J. biol. Chem.* **221**, 593.
Yang, C. S. & McCormick, D. B. (1967). *J. Nutr.* **93**, 445.

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