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Metabolism of riboflavin: reduction of 7,8-dimethyl-10-formylmethylisoalloxazine by an enzyme in liver

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1. The riboflavin metabolite, 7,8-dimethyl-10-formylmethylisoalloxazine, has been shown to be reduced to the corresponding alcohol, 7,8-dimethyl-10-(2′-hydroxyethyl)isoalloxazine, by an enzyme present in the soluble fraction of liver and kidney.

2. The enzyme is present in these organs in a number of species but is especially prevalent in the tissues of ruminants. It appears to be different from the normal hepatic alcohol dehydrogenase in that it uses NADPH rather than NADH as cofactor.

When riboflavin is eaten by ruminants or man in quantities much larger than a normal daily requirement, the urinary concentrations of the vitamin are considerably increased and, in addition, various metabolites of the vitamin make their appearance in the urine (Owen & West, 1970). Three of these fluorescent metabolites have been identified and evidence has been presented to demonstrate that they are formed by the degradative action of the symbiotic bacteria in the gut of animals (Owen 1962; Owen & West, 1970). The major metabolite occurring in ruminant urine is 7,8-dimethyl-10-(2′-hydroxyethyl)isoalloxazine (hydroxyethylflavin) accompanied by only comparatively small amounts of 7,8-dimethyl-10-formylmethylisoalloxazine (formylmethylflavin) and yet, when rumen contents are incubated in vitro, the latter product is initially formed in the greatest amounts. The reduction of formylmethylflavin to hydroxyethylflavin bears an obvious resemblance to the normal acetaldehyde → ethanol reduction carried out by hepatic alcohol dehydrogenase and this suggested that the comparative lack of isoalloxazine aldehyde in urine might be due to such a reduction occurring within mammalian tissues.

It is the purpose of the present report to demonstrate that such a reduction does occur in the liver and kidney tissues of a number of species, and that the enzyme which catalyses the reaction is present in the cytosol fraction of such tissues. In addition, evidence is presented to show that this reduction is not due to the action of the normal NADH-coupled hepatic alcohol dehydrogenase.

EXPERIMENTAL

Materials

Riboflavin was purchased from Koch–Light Laboratories Ltd, Colnbrook, Bucks., and crystalline yeast and liver alcohol dehydrogenases (alcohol: NAD oxidoreductase; *EC* 1.1.1.1) from Boehringer Mannheim Ltd, London. For thin-layer chromatography, Camag Kieselgel D5 (Griffin & George, East Kilbride, Scotland) was used.

The substrate, formylmethylflavin, was prepared according to the method of Fall & Petering (1956) and was purified to an orange crystalline solid by the method of Owen & West (1968). For the assays a solution (about 100 μmol) was prepared in 50 mM-tris–HCl buffer (pH 7·2) and the exact concentration of the substrate was determined by comparing its fluorescence with that of a riboflavin standard solution in a Locarte fluorimeter (The Locarte Company, Emperor's Gate, London).

The procedures of Fall & Petering (1956) were also used to prepare a sample of hydroxyethylflavin and its acetoxy derivative.

Assay methods

Reactions were carried out in heavy-walled centrifuge tubes and in Thunberg tubes. Incubation was in a water-bath at 37° for 2 h unless stated otherwise. In view of the possibility that the flavins may have been absorbed on to precipitated protein and because of the presence of perchloric acid interfering with thin-layer chromatographic analysis, no attempt was made to stop the reaction by addition of any of the usual protein precipitating reagents. The silica-gel chromatoplates each had marker spots of synthetic hydroxyethylflavin applied before development in a chloroform–methanol (9:1, by volume) solvent system. The yellow-fluorescing band corresponding to the required product was scraped from the plate and the isoalloxazine was eluted from the silica with a mixture of ethanol and water (4:1, by volume). The amount of product was determined by measuring the fluorescence of the eluate against that of a riboflavin standard solution prepared in the same solvent mixture. The identity of the product was confirmed by its chromatographic behaviour and by that of its acetoxy derivative (Owen & West, 1970) in the solvent systems (*a*) chloroform–methanol (9:1, by volume), (*b*) butanol–acetic acid–water (4:1:5, by volume, upper phase), (*c*) chloroform–pyridine–acetic acid–methanol (8:1:1:1, by volume) and (*d*) chloroform–butanol–acetic acid (5:4:1, by volume).

Preparation of biological materials

All operations were conducted between 0° and 4° unless otherwise indicated. Liver and kidney samples were obtained from freshly slaughtered cattle, sheep and goats, and a suitable piece of each tissue was excised. The tissue was blotted dry, weighed, and added to three to five times its volume of ice-cold 0·25 M-sucrose containing 10 mM-magnesium chloride, 75 mM-potassium chloride, and 35 mM-tris–HCl buffer, pH 7·2. The piece of tissue was then cut into small pieces with a pair of scissors and homogenized in this medium in an all-glass apparatus of the Potter–Elvehjem type using only six strokes of the plunger to minimize the rupture of mitochondria. The homogenate was centrifuged at 750 *g* for 10 min. The supernatant fraction was removed and subjected to a further centrifugation at 14000 *g* for 10 min. The supernatant fraction was again removed and centrifuged at 105000 *g* for 60 min to give a clear supernatant solution. The pellets from the three centrifugations were each resuspended in the isolation medium and were made up to a volume equal to that of the original homogenate. The mitochondrial and microsomal fractions were cooled in ice and exposed to oscillations at 8 mA for 5 min from an 100 W sonic oscillator

(Measuring & Scientific Equipment Ltd, Buckingham Gate, London) equipped with a 9·5 mm probe. The three pellet fractions (nuclei, mitochondria and microsomes) and the supernatant fraction were all assayed qualitatively for their ability to reduce formylmethylflavin to hydroxyethylflavin. The supernatant fraction was used either directly or after dialysis overnight against two changes of 20–30 volumes of 50 mM-tris–HCl buffer, pH 7·2, at 4°.

The effects of Mg^{2+} and Ca^{2+} on the reaction were assayed quantitatively, using a homogenate prepared from a sample of sheep liver, and the same tissue sample was used to study the optimum pH and the cofactor requirements. In addition, for the determination of the cofactor requirements, triethanolamine–HCl buffer, pH 7·6, replaced the tris–HCl buffer in the isotonic sucrose medium and the high-speed supernatant fraction was dialysed against two changes of 100 mM-triethanolamine–HCl buffer, pH 7·6.

RESULTS

Homogenates of liver and kidney samples from ruminants (goats, sheep and cattle) were capable of reducing formylmethylflavin to the corresponding alcohol hydroxyethylflavin. No hydroxyethylflavin was produced when either the substrate or the homogenate was omitted from the reaction mixture.

No hydroxyethylflavin was produced when the homogenates were heated at 100° for 2 min before addition to the assays, indicating that the reduction involved a heat-labile material. The ability of crude extracts to reduce formylmethylflavin was lost on overnight storage at 4° but not at −15°. Dialysis of a crude extract against 50 mM-tris–HCl buffer, pH 7·2, for 20 h at 0° also removed its ability to reduce formylmethylflavin, but this was restored when the diffusate was added to the reaction mixture. When the crude liver homogenates were separated into mitochondrial and other fractions by differential centrifugation, only the soluble portion of the cellular material showed any activity. The microsomal and mitochondrial fractions were assayed both before and after being subjected to ultrasonic disintegration, but no activity was observed in either fraction.

Dialysis of the soluble supernatant fraction, from a homogenate of a sample of sheep liver, against 35 mM-tris–HCl buffer, pH 7·2 containing 10 mM-magnesium chloride for 20 h at 4° did not cause loss of enzymic activity. However, when magnesium chloride was omitted from the surrounding buffer, dialysis for the same period caused loss of activity, which could be restored by the addition of magnesium ions to the non-diffusible material (Table 1). Calcium could substitute for magnesium (Table 1) but the addition to the assay mixture of manganese or zinc caused precipitation of protein and loss of enzymic activity.

Optimum pH

The optimum pH for the conversion of formylmethylflavin was determined using the soluble 105000 *g* supernatant fraction of a sheep liver homogenate that had been dialysed overnight against 20 mM-tris–HCl buffer, pH 7·2. Tris–HCl and tris–maleate–NaOH buffers were used to cover the pH range 5·8–8·2. The results, plotted

Table 1. *Effect of magnesium and calcium ion concentrations on the reduction of* 7,8-*dimethyl*-10-*formylmethylisoalloxazine* (*formylmethylflavin*) *to* 7,8-*dimethyl*-10-(2′-*hydroxyethyl*)*isoalloxazine* (*hydroxyethylflavin*)

Divalent ion	Concentration (μmol)	Hydroxyethylflavin formed (nmol)
None	—	0·0
Mg^{2+}	2·3	0·8
Mg^{2+}	1·2	1·1
Mg^{2+}	0·3	0·8
Ca^{2+}	2·3	0·9
Ca^{2+}	1·2	0·8
Ca^{2+}	0·3	1·1

Each 0·4 ml reaction mixture contained 200 μl of a 105000 *g* supernatant fraction from a homogenate of sheep liver which had been dialysed against 50 mM-tris–HCl buffer, pH 7·2, 2·1 nmol formylmethylflavin, 20 μmol tris–HCl buffer, pH 7·2, and magnesium or calcium at the concentrations shown in the table. Incubation was at 37° for 2 h.

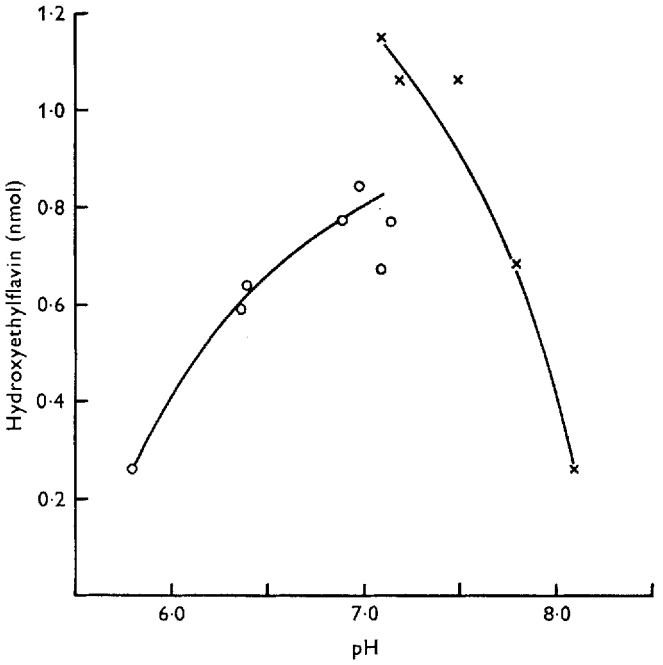

Fig. 1. Variation in formation of 7,8-dimethyl-10-(2′-hydroxyethyl)isoalloxazine (hydroxyethylflavin) with pH. Each 0·4 ml reaction mixture contained 200 μl of a dialysed 105000 *g* supernatant fraction from a homogenate of sheep liver, 2·1 nmol formylmethylflavin, 1·15 μmol magnesium chloride and 20 μmol tris–HCl (-○-) or tris–maleate–NaOH (-×-) buffer at the pH indicated. Incubation was at 37° for 2 h.

Table 2. *Cofactor requirements for maximum enzymic activity in the reduction of* 7,8-*dimethyl*-10-*formylmethylisoalloxazine* (*formylmethylflavin*) *to* 7,8-*dimethyl*-10-(2′-*hydroxyethyl*) *isoalloxazine* (*hydroxyethylflavin*)

Addition	Hydroxyethylflavin produced (nmol)
None	1·2
NADPH (100 nmol)	4·8
NADH (100 nmol)	1·4
NADH (100 nmol), ATP (50 nmol)	2·4
Hydrogen	1·2
Nitrogen	1·2

Each reaction mixture contained 200 μl of a dialysed 105000 *g* supernatant fraction from a sheep liver homogenate, 5 nmol formylmethylflavin, 1·15 μmol magnesium chloride, 50 μmol triethanolamine–HCl buffer, pH 7·6, cofactor additions as shown, and distilled water to a total volume of 0·55 ml. Incubations were at 37° for 1 h.

in Fig. 1, show that the pH optimum was around 7·0, although the change of buffer makes it difficult to locate more closely.

Non-reduction with liver alcohol dehydrogenase

When formylmethylflavin was incubated in phosphate buffer, pH 7·0, with a thirty-five-fold excess of NADH in the presence of crystalline horse liver alcohol dehydrogenase, the change in extinction coefficient at 340 nm indicated that approximately 2% of the pyridine nucleotide was oxidized (equivalent to 76% of the isoalloxazine present). However, when the assay mixture was analysed by thin-layer chromatography no fluorescent compound other than the starting material was present. The ratio of enzyme to substrate was varied over the range 1:1 to 1:60, but under no circumstances was any hydroxyethylflavin detected in the assays. Although yeast alcohol dehydrogenase has a wider specificity than the liver enzyme it gave identical results when assayed under the same conditions and it was obvious that formylmethylflavin was not a substrate for these enzymes.

Cofactor requirements

By analogy with liver alcohol dehydrogenase, the most obvious cofactor to examine was NADH. However, as can be seen in Table 2, the addition of this pyridine nucleotide in twentyfold excess over the substrate caused only a slight increase in the reduction of the formylmethylflavin in comparison with the result achieved when no cofactor was present in the assay. When ATP was included with NADH in the assay mixture the amount of hydroxyethylflavin formed was markedly increased. However, over the same period of incubation, the presence of NADPH in the assay mixture ensured essentially complete reduction of the formylmethylflavin to the corresponding alcohol. No attempt was made to try to correlate the oxidation of the pyridine nucleotides with the formation of hydroxyethylflavin in view of the result achieved in the attempted

Table 3. *Variation of formation of 7,8-dimethyl-10-(2'-hydroxyethyl)-isoalloxazine (hydroxyethylflavin) with species*

Animal	Hydroxyethylflavin produced (nmol/g tissue) Liver	Kidney
Pig	0	0
Rat	2·6	0
Guinea-pig	6·8	0
Chicken	10·5	9·3
Mouse	10·5	9·3
Rabbit	8·3	19·8
Goat	25·5	22·9
Sheep	29·8	Not measured

The 105000 *g* supernatant fraction from a homogenate of each tissue was dialysed against 50 mM-tris–HCl buffer, pH 7·2. Each 0·4 ml reaction mixture contained 200 μl of a dialysed 105000 *g* supernatant fraction of the tissue indicated, 2·1 nmol 7,8-dimethyl-10-formylmethylisoalloxazine, 1·15 μmol magnesium chloride and 20 μmol tris–HCl, pH 7·2. Incubation was at 37° for 1 h.

reduction of formylmethylflavin with horse liver alcohol dehydrogenase when oxidation of the pyridine nucleotide had occurred without concomitant formation of hydroxyethylflavin.

Exclusion of oxygen from the assay by either nitrogen or hydrogen did not stimulate the activity of the enzyme.

Species variation

Ruminant and non-ruminant liver have different patterns of enzyme activity (Ballard, Hanson & Kronfeld, 1969), and to determine whether the enzyme responsible for the reduction of formylmethylflavin was specific to ruminants, liver and kidney samples were obtained from some other species (Table 3). After homogenization in tris–HCl buffer, pH 7·2, both the 105000 *g* supernatant fractions and the resuspended particulate fractions from each tissue were assayed for activity. None of the particulate suspensions showed any signs of enzymic activity, in confirmation of the results previously obtained with the ruminant tissue samples. In contrast, most of the 105000 *g* supernatant fractions were active, although the activity as measured by hydroxyethylflavin production per g tissue varied from species to species (Table 3). The liver from the goat was three times as active as the same tissue from the rabbit, whereas the activity in the kidney from these two species was similar. In the samples of pig liver and kidney that were examined, the enzyme activity was too small to be measured by our techniques.

DISCUSSION

Riboflavin is not degraded by animal tissues and if injected intravenously into an animal it is excreted unchanged in the urine. If, however, formylmethylflavin is injected in the same manner it is partially converted into hydroxyethylflavin within the animal so that both materials appear in the milk and urine (Owen & West, 1970).

The evidence presented here demonstrates that the liver and kidneys of ruminants and of some other species contain an enzyme that is capable of reducing this isoalloxazine aldehyde to the corresponding alcohol. The fact that the tissues of ruminants and of the rabbit show the highest activity of this enzyme agrees well with the fact that hydroxyethylflavin can be detected in the urine of these animals when on normal feeding regimens (Owen & West, 1970; Owen, West & Coates, 1970). These species rely largely on bacterial fermentations for digestion of food materials and the development of enzymes capable of dealing with bacterial metabolic products would be an expected adaptation.

The reduction of an aldehyde to an alcohol is a common process in animal tissues, and the NAD-dependent alcohol dehydrogenases in liver are well characterized. However, from the results shown here it can be seen that the isoalloxazine aldehyde is not a substrate for this particular enzyme. The oxidation of the small amount of NADH in this assay is not explainable at present but, in view of the oxidation–reduction properties of isoalloxazines, it is possible that the hydrogen was transferred to the heterocyclic nucleus of the flavin substrate rather than used to reduce the aldehyde group (Suelter & Metzler, 1960). The isoalloxazine nucleus is easily reoxidized and thin-layer chromatographic analysis would thus show no detectable change.

The enzyme present in the extracts obtained from sheep liver appears to use NADPH as a cofactor in preference to NADH. The increased formation of hydroxyethylflavin that occurred when ATP was added to the reaction mixture containing NADH supports this hypothesis, since the addition of both these nucleotides to the extracts would allow the generation of NADPH *in situ*.

An NADP-dependent alcohol dehydrogenase has been previously reported in wheat germ (Stafford & Vennesland, 1953), in *Leuconostoc mesenterioides* (DeMoss, 1955) and as a contaminant in a crystalline preparation of the NAD-linked enzyme (Sable, 1952). It may be that the enzyme here reported is similar to these, but initial attempts to demonstrate the reversibility of the reaction have not been successful. The attempts to demonstrate the oxidation of hydroxyethylflavin to formylethylflavin were made at an alkaline pH, at which formylmethylflavin is unstable, and it could be argued that the reverse reaction does occur but that the product is degraded before it can be detected. However, at alkaline pH, formylmethylflavin is degraded to 7,8,10-trimethyl isoalloxazine (lumiflavin) and, although this product has essentially the same fluorescent properties as the substrate, it is easily identified on thin-layer chromatographic analysis. No fluorescent compounds other than the starting material were detected in the assay mixtures.

It is unlikely the formylmethylflavin is the sole substrate for this enzyme but determination of the substrate specificity of the enzyme will be dependent on the availability of a much purer sample of this material.

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