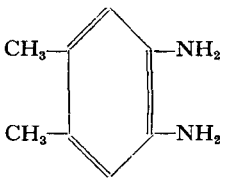
Metabolic interrelationships of dietary riboflavin and vitamin B_{12} in the rat

By W. V. LAVATE and A. SREENIVASAN

Department of Chemical Technology, University of Bombay, Matunga Road, Bombay

(*Received* 14 *April* 1959—*Revised* 30 *June* 1959)

Both riboflavin and vitamin B_{12} have in common a 1,2-dimethyl-4,5-diaminobenzene moiety (substance I), which could well be a biogenetic precursor of these two vitamins. Although substance I has not been demonstrated to be in the pathway for the biosynthesis of these vitamins, a considerable amount of evidence is available in favour of such a view.



Substance I, 1,2-dimethyl-4,5-diaminobenzene

In micro-organisms, substance I exhibited some small degree of riboflavin activity for *Lactobacillus casei* and also had some vitamin B_{12} potency for *Euglena gracilis*, as well as for some other organisms requiring vitamin B_{12} (Woolley, 1950, 1951). Addition of substance I to cultures of *Bacillus megatherium* stimulated vitamin B_{12} synthesis (Woolley, 1950). The precursor activity of substance I has been further suggested by the observation that an analogue, 1,2-dichloro-4,5-diaminobenzene, competitively inhibited synthesis by *B. megatherium* of riboflavin and vitamin B_{12} from substance I (Woolley, 1950). Addition of cobalt, which would normally stimulate synthesis of vitamin B_{12} by *B. megatherium*, with this analogue was found to aggravate the inhibition of the synthesis (Woolley, 1950). Addition of substance I, or of the substances that closely resemble it, 5,6-dimethylbenziminazole and *o*-phenylenediamine, is known to stimulate synthesis of vitamin B_{12} by *Streptomyces olivaceous* (Ganguly & Roy, 1956) and by *Streptomyces griseus* (Dulaney & Williams, 1953). Riboflavin and 5,6-dimethylbenziminazole have been shown to cause a small but significant increase in synthesis of vitamin B_{12} by *Escherichia coli* (cf. Ford & Hutner, 1955).

Added support for the view that biosynthesis of riboflavin and vitamin B_{12} may involve a common precursor is provided by observations that in media supplemented with cobalt there is an increased synthesis of riboflavin by *Ashbya gossypii* (Hickey, 1954), and that flavinogenesis by various micro-organisms synthesizing riboflavin was stimulated by additions of certain purines and amino acids (Ford & Hutner, 1955). Since single-carbon fragments may take part in the biosynthesis of riboflavin much in

the same way as in the synthesis of purines and pyrimidines, this consideration may be taken to provide presumptive evidence that vitamin B_{12} can participate in the synthesis and mobilization of C_1 fragments (Ford & Hutner, 1955). It may also help in explaining why synthesis of riboflavin by *A. gossypii* (Smiley, Sobolov, Austin, Rasmussen, Smith, Van Lanen, Stone & Boruff, 1951) and by *Eremothecium ashbyii* (W. V. Lavate, unpublished) is invariably accompanied by synthesis of small but significant amounts of vitamin B_{12}-active compounds.

In animals, Emerson, Brink, Holly, Koniuszy, Heyl & Folkers (1950) observed that substance I and 5,6-dimethylbenziminazole showed vitamin B_{12} activity when administered to rats maintained on a diet devoid of animal-protein products and containing thyroid powder, but that these two compounds were required for the purpose in larger amounts than vitamin B_{12}. It was observed further (Emerson, Holly, Shunk, Brink & Folkers, 1951) that a higher derivative, α-ribazole (1-α-D-ribofuranoside-5,6-dimethylbenziminazole), exhibited vitamin B_{12} activity of the same order as vitamin B_{12} itself. However, a riboflavin-like derivative, 1-D-ribityl-5,6-dimethylbenziminazole, possessed much less vitamin B_{12} activity. Similar results were reported by Cooperman & Tabenkin (1951) with riboflavin, and some of the above compounds tested orally or by intraperitoneal injection. Hartman, Dryden & Cary (1949, 1951) noted that large amounts of dietary riboflavin may promote intestinal synthesis of vitamin B_{12} in rats.

Pronounced lowering of the concentration of vitamin B_{12} in liver, kidney and heart has been observed in riboflavin deficiency (Bhagwat & Sohonie, 1954, 1955). Similarly, diminution in the riboflavin content of the liver (Bhagwat & Sohonie, 1955) and decrease in activities of some liver dehydrogenases (Bhagwat & Sohonie, 1955; Murthy, Desikachar & Swaminathan, 1956; Williams, Monson, Sreenivasan, Dietrich, Harper & Elvehjem, 1953) have been reported in vitamin B_{12} deficiency.

This survey of the available information on the interrelation of riboflavin and vitamin B_{12} would seem to indicate that in micro-organisms there is good evidence that riboflavin and vitamin B_{12} may have common biogenetic pathways. However, for animals, information about the extent and nature of this interrelationship is not adequate, and the position is complicated by microbial activities in the intestinal tract.

In this paper, some experiments are described with rats rendered deficient in either of these vitamins.

EXPERIMENTAL

Animals and management

Experiment 1. Twenty-four weanling rats (Wistar strain) of both sexes were reared for 4 weeks on a basal diet free from riboflavin and vitamin B_{12} of the percentage composition: ethanol-extracted casein 18, maize starch 60, vitaminized sucrose 11, vitaminized sesame oil 7, and salt mixture (U.S. Pharmacopoeia, XIV, 1950) 4. Vitamins A, D and E (α-tocopherol) were provided with the sesame oil and the others with the sucrose. The amounts of vitamins supplied per kg diet were: thiamine hydrochloride 6 mg, nicotinic acid 30 mg, calcium pantothenate 20 mg, pyridoxine hydrochloride 6 mg, folic acid 5 mg, biotin 1 mg, *p*-aminobenzoic acid 100 mg, choline chloride

500 mg, inositol 500 mg, menaphthone 10 mg, vitamin A 5000 i.u., vitamin D 50 i.u. and α-tocopherol 50 mg.

At the end of this initial depletion period, growth had ceased almost completely and other characteristic signs of riboflavin deficiency had appeared. At this stage, the animals were divided into four similar groups. One group continued to receive the basal diet, and the remaining three were placed on the basal diet supplemented with 200 μg/kg vitamin B_{12} or 10 mg/kg riboflavin or both.

The animals were fed *ad lib.* for 4 weeks, and their weight was recorded at weekly intervals. All the animals were then killed under ether anaesthesia, to obtain samples of blood and liver. Blood drawn from the hepatic portal vein was immediately citrated. The livers were chilled and made into 20% homogenates with ice-cold distilled water in a Potter–Elvehjem glass homogenizer.

Experiment 2. Twenty-four animals were reared during the pre-experimental period of 4 weeks on the basal diet supplemented with 0·15% iodinated casein (Protomone, Cerophyl Laboratories, Kansas City, Mo., U.S.A.) to accelerate the depletion of vitamin B_{12} reserves. The various experimental diets given for the next 4 weeks did not contain iodinated casein, but included 1·5% succinylsulphathiazole to suppress intestinal synthesis. The basal diet, grouping of animals and the experimental diets were otherwise the same as in Expt 1.

This procedure for induction and maintenance of vitamin B_{12} deficiency was not attended by complications such as are likely to result from prolonged administration of diets with iodinated protein (cf. Fatterpaker, Lavate, Mulgaonkar, Noronha, Rege, Tipnis & Sreenivasan, 1959). The vitamin supplements provided were adequate to meet the needs of the animals (cf. Ershoff, 1948).

Analytical methods

Vitamin B_{12} was liberated from liver and blood by treatment with papain (British Drug Houses Ltd), and was assayed in liver samples with *E. gracilis* (Hoff-Jørgensen, 1954) and in blood samples with *Lactobacillus leichmannii* A.T.C.C. 7830 by a turbidimetric modification of the U.S.P. method (Rege, 1953). Flavinadenine dinucleotide (FAD) and total riboflavin in liver were determined fluorimetrically by the procedure of Bessey, Lowry & Love (1949).

Xanthine-oxidase activity of liver was estimated manometrically by the method of Dhungat & Sreenivasan (1954).

To determine total protein in liver, 2·5 ml of the 20% homogenate (equivalent to 500 mg of fresh tissue) was taken in a tared centrifuge tube to which 7 ml of a 10% (w/v) solution of trichloroacetic acid were added. The tissue was well dispersed in the trichloroacetic-acid solution with a glass rod, centrifuged and the supernatant liquid was poured off. The treatment with trichloroacetic acid was repeated twice and was then followed once by treatment with 5 ml of 95% ethanol, two further treatments with 5 ml portions of a 3:1 mixture of 95% ethanol and diethyl ether, and a final treatment with 5 ml of diethyl ether. The residue was thoroughly mixed every time and centrifuged for 10 min at 800 ***g***. The residue left finally in the centrifuge tube was dried at 80° for about 18 h and weighed to give the weight of protein.

It was observed that the results obtained by this procedure agreed well with those obtained by the Kjeldahl method, as has also been reported by Osborn, Felts & Chaikoff (1953) with a similar procedure.

Table 1. *Expt 1. Effect on rats depleted of riboflavin and vitamin B_{12} of a diet free from both vitamins or supplemented with one or with both*

(Mean values with their standard errors; six animals/group)

Diet	Weight gain in 4 weeks (g)	Vitamin B_{12} Blood (mμg/ml)	Vitamin B_{12} Liver (mμg/g)	Liver flavinadenine dinucleotide (μg/g)	Total liver riboflavin (μg/g)	Liver xanthine-oxidase activity (μl O_2/h/g)	Total liver protein (g/100 g)
Basal*	15·0 ± 5·0	1·30 ± 0·15	56·0 ± 3·0	6·4 ± 0·30	8·2 ± 0·20	111·5 ± 18·5	14·8 ± 0·5
Basal+vitamin B_{12}†	18·0 ± 6·0	1·20 ± 0·10	75·0 ± 3·5	6·1 ± 0·25	9·8 ± 0·35	112·0 ± 22·5	15·3 ± 0·7
Basal+riboflavin‡	110·0 ± 10·0	1·35 ± 0·10	82·0 ± 4·0	17·7 ± 0·75	31·0 ± 1·50	250·0 ± 12·5	17·1 ± 0·9
Basal+vitamin B_{12}†+riboflavin‡	125·0 ± 10·0	1·45 ± 0·15	93·0 ± 4·5	20·1 ± 0·90	30·0 ± 1·20	279·0 ± 10·0	17·4 ± 0·6

* Free from riboflavin and vitamin B_{12}. † 200 μg/kg. ‡ 10 mg/kg.

RESULTS

The results of the first experiment are reported in Table 1. It may be seen that severe riboflavin deficiency was produced in animals reared on diets devoid of this vitamin. The rats grew little during the 4 weeks and had considerably less FAD, total riboflavin and xanthine oxidase in their livers than the controls. However, animals on the diets free from vitamin B_{12} did not develop appreciable deficiency, and it appeared that considerable intestinal synthesis of vitamin B_{12} had occurred. Since the production of vitamin B_{12} deficiency was not satisfactory in this experiment, not much could be ascertained about the effects of associated deficiencies of these two vitamins.

The results of the second experiment are summarized in Table 2. The values for liver vitamin B_{12} in the animals on the diets free from vitamin B_{12} show that a satisfactory deficiency was produced. The manifestations of riboflavin deficiency were almost the same as in Expt 1.

Table 2. *Expt 2. Effect on rats depleted of riboflavin and vitamin B_{12} of a diet containing 1·5 % succinylsulphathiazole and free from both vitamins or supplemented with one or with both*

(Mean values with their standard errors; six animals/group)

Diet	Weight gain in 4 weeks (g)	Vitamin B_{12} Blood (mμg/ml)	Vitamin B_{12} Liver (mμg/g)	Liver flavinadenine dinucleotide (μg/g)	Total liver riboflavin (μg/g)	Liver xanthine-oxidase activity (μl O_2/h/g)	Total liver protein (g/100 g)
Basal*	14·0 ± 6·0	1·15 ± 0·25	45·0 ± 3·0	7·3 ± 0·50	11·1 ± 0·40	162·5 ± 42·5	12·9 ± 1·0
Basal+vitamin B_{12}†	16·0 ± 4·0	1·00 ± 0·20	82·0 ± 7·0	7·7 ± 0·80	12·0 ± 0·60	152·5 ± 50·0	13·5 ± 0·8
Basal+riboflavin‡	95·0 ● 15·0	1·55 ± 0·15	42·0 ± 5·0	15·5 ± 0·50	28·2 ± 0·80	277·5 ± 40·0	16·4 ± 0·4
Basal+vitamin B_{12}†+riboflavin‡	108·0 ± 12·0	1·40 ± 0·30	108·0 ± 8·0	20·2 ± 0·85	29·5 ± 0·50	360·5 ± 49·5	16·0 ± 0·7

* Free from riboflavin and vitamin B_{12}. † 200 μg/kg. ‡ 10 mg/kg.

There was some mortality in riboflavin-deficient animals in both experiments, due to the severity of the deficiency. Depression of growth was not alleviated to any significant extent by administration of vitamin B_{12}. Though full growth occurred only when both riboflavin and vitamin B_{12} were present, deficiency of vitamin B_{12} alone caused only a slight retardation of growth.

In a combined deficiency of riboflavin and vitamin B_{12}, growth retardation and

decreases in liver contents of total protein, xanthine oxidase, FAD, non-FAD and total riboflavin, and vitamin B_{12} as well as in blood vitamin B_{12} were, in general, more pronounced than those in the single deficiency of either vitamin.

Addition of vitamin B_{12} to the basal diet improved the liver vitamin B_{12} level, but caused no significant alterations in blood vitamin B_{12} or in other liver constituents.

DISCUSSION

In groups on diets adequate in vitamin B_{12}, liver levels of this vitamin were influenced by the intake of riboflavin, which suggests that lack of riboflavin adversely affected the metabolism and storage in liver of vitamin B_{12}.

With a deficiency of vitamin B_{12} alone, the levels of liver protein and riboflavin were nearly normal. However, the contents of liver FAD and of its xanthine-oxidase activity were only partly restored. A non-specific decrease in the xanthine-oxidase activity of the liver in vitamin B_{12}-deficient rats has also been reported by Williams *et al.* (1953). This observation appears to be of interest, since it points to a probable function of vitamin B_{12} in the biosynthesis of FAD from non-FAD riboflavin. In rat liver, FAD synthesis from riboflavin occurs as follows (Schneider, 1955):

$$\text{Riboflavin} + \text{adenosine triphosphate (ATP)} \rightarrow \text{flavinadenine mononucleotide (FMN)} + \text{adenosine diphosphate}$$

$$\text{FMN} + \text{ATP} \rightarrow \text{FAD} + \text{pyrophosphate.}$$

The site of synthesis is exclusively the soluble fraction of liver, and the FAD thus synthesized is to a large extent (up to 65 %) stored in mitochondria (Schneider, 1955). Other observations (unpublished observations of U. Marfatia, H. P. Tipnis, D. V. Rege and A. Sreenivasan in this laboratory) have shown that the decrease in liver content of FAD in vitamin B_{12} deficiency is paralleled by a decrease in mitochondrial content; this work has also shown that the conversion in vivo of administered riboflavin into FAD is impaired by a deficiency of vitamin B_{12}, and is restored to normal by supplementation with the vitamin. A protection exerted by prior administration of vitamin B_{12} against certain metabolic derangements in the rat caused by experimental thyrotoxicosis or liver injury was reported by Kasbekar, Lavate, Rege & Sreenivasan (1959*a*, *b*). This protection was evidenced primarily by reduction in the losses of intramitochondrial components including nucleotides, vitamin B_{12} and glutathione (Kasbekar & Sreenivasan, 1956; Kasbekar, Rege & Sreenivasan, 1959), which occur under the conditions of stress studied. It seems likely, therefore, that the decreased synthesis of FAD in vitamin B_{12} deficiency may be accompanied by a release of intramitochondrial FAD. However, further work is necessary to ascertain the specificity of such relationships of various stress or deficiency states to the changes, qualitative and quantitative, in cell components.

In animals deficient only in vitamin B_{12} (Expt 1), considerable increase in liver vitamin B_{12} occurred, even though the vitamin was absent from the diet. When a sulpha drug was present in the same diet (as in Expt 2) no such increase in the liver vitamin B_{12} was observed. Since the diets were adequate in riboflavin, these results suggest that, in the absence of the sulpha drug, dietary riboflavin could induce profuse

synthesis of vitamin B_{12} in the intestine, a part, at least, being absorbed by the host animal. The observations support the conclusion of Hartman *et al.* (1949, 1951) that dietary riboflavin induces intestinal synthesis of vitamin B_{12}-active materials. Their conclusion was, however, based only on observations on growth.

In uncomplicated riboflavin deficiency, dietary vitamin B_{12} was not effective in improving the riboflavin status of the animals, which indicates that dietary vitamin B_{12} does not support intestinal synthesis of riboflavin or that such synthesis is inadequate. Thus, liver levels of FAD and riboflavin of animals deficient only in riboflavin were not different from those of the group deficient in both riboflavin and vitamin B_{12}, and were considerably lower than those of animals receiving riboflavin alone or together with vitamin B_{12}.

The activity of riboflavin in promoting intestinal synthesis of vitamin B_{12} need not necessarily imply that the vitamin is actually broken down to provide a nucleotide precursor of the vitamin B_{12} molecule. An alternative explanation could be that a common precursor of riboflavin and vitamin B_{12} is normally available in limiting amounts, and is used preferentially for the synthesis of riboflavin. The provision of preformed riboflavin might then spare such a precursor for the synthesis of vitamin B_{12} (Ford & Hutner, 1955). This suggestion may also explain why vitamin B_{12} is not broken down in the intestine to provide the benziminazole moiety for riboflavin synthesis.

SUMMARY

1. Twenty-four weanling rats were fed for 4 weeks on a diet free from riboflavin and vitamin B_{12}. They were then divided into four groups; one received for the next 4 weeks the basal diet alone and the other three the basal diet supplemented with 200 μg vitamin B_{12}/kg or 10 mg riboflavin/kg or both. A second lot of twenty-four weanling rats was treated in the same way except that the diet given in the first 4 weeks was supplemented with 0·15 % iodinated casein and each experimental diet with 1·5 % succinylsulphathiazole.

2. The effects of deficiencies of riboflavin and vitamin B_{12} on the liver storage of these two vitamins were studied. The protein content and xanthine-oxidase activity of the liver were also examined. In addition, the influence of the intestinal microflora upon these changes was investigated.

3. In the riboflavin-deficient rats there was a pronounced fall in the liver content of vitamin B_{12} and protein. In vitamin B_{12}-deficient rats, the flavinadenine-dinucleotide content and xanthine-oxidase activity, but not the total riboflavin content, of the liver were decreased.

4. Dietary riboflavin appeared to induce profuse intestinal synthesis of vitamin B_{12}, which was checked by addition of succinylsulphathiazole to the diet. Dietary vitamin B_{12} apparently did not stimulate intestinal synthesis of riboflavin, but appeared to influence in some way the biosynthesis of FAD from non-FAD riboflavin in animal tissues.

One of us (W.V.L.) wishes to thank the Ministry of Education, Government of India, for a research fellowship, during the tenure of which this work was carried out.

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