Studies on Relationships Between Riboflavin and Folate

Relationships between riboflavin and folate were demonstrated first by NELSON et al.[1]. New-borns of riboflavin-deficient rats presented congenital malformations similar to those induced by folate deficiency. Afterwards MILLER et al.[2] showed a decrease in the liver levels of folate and citrovorum factor in riboflavin-deficient pregnant rats.

In order to obtain additional data regarding these relationships, the excretion of formiminoglutamic acid (FIGlu) and 4-amino-5-imidazolecarboxamide (AIC) in riboflavin-deficient rats was investigated. FIGlu degradation is dependent on the tissue storage of folate coenzymes[3] as well as the conversion of AIC into inosinic acid[4]. Consequently, the estimation of urinary FIGlu and AIC is a sensitive and specific index of folate deficiency or of impaired function of folate coenzymes.

Liver formiminoglutamate[5] H_4 folate-5-formimino transferase (FIGlu transferase) (EC 2.1.2.5) and aminoimidazolecarboxamide ribotide transformylase (AICR-transformylase) were also measured.

Materials and methods. Male weanling albino rats of Wistar strain were divided into 2 groups, and fed ad libitum on a riboflavin-deficient diet[6], and the same diet supplemented with 30 mg of riboflavin per kg of diet respectively. After 60 days of treatment the animals received by stomach-tube 1 ml of 0.1 *M* L-histidine and 2 ml of sterile 0.9% NaCl by i.p. injection per 100 g of body weight.

Then the rats were placed in individual metabolic cages and urine was collected for 24 h in bottles containing 0.5 ml of 0.5 *N* HCl. FIGlu was determined measuring at 365 nm the 5,10-CH=H_4 folate formed incubating at 25 °C for 30 min samples of urine with suitable amounts of FIGlu-transferase, HCNH-H_4 folate cyclodeaminase, H_4 folate and 2-mercaptoethanol[7]. AIC was determined by the method of BRATTON and MARSHALL[8] in aliquots of urine passed through a column of Biorad AG 2 × 8, 200 to 400 mesh, Cl^- form, to remove interfering materials[9].

For the estimation of FIGlu-transferase, the rats were sacrificed and liver acetone powders were prepared. The powder was extracted with 10 volumes of H_2O at 2 °C, stirring for 15 min. The suspension obtained was then centrifuged at 20,000 *g* for 15 min and the supernatant was added with solid ammonium sulphate (0.2 g/ml of solution). After 2 h the precipitate was collected by centrifuging at 20,000 *g* and suspended in a suitable amount of 0.2 *M* sodium acetate; the solution was allowed to stand at 2 °C for 16 h. The FIGlu transferase activity was measured, incubating for 30 min at 25 °C an aliquot enzyme solution with FIGlu, H_4 folate and phosphate buffer pH 7.2. The 5,10-CH=H_4 folate formed was measured spectrophotometrically at 355 nm[10].

For the estimation of AICR transformylase the acetone powder was extracted at 2 °C for 30 min with 0.01 *M* *Tris*-HCl buffer pH 7.4. The suspension was centrifuged at 13,000 *g* for 10 min, and the supernatant was added with ammonium sulphate (0.2 g/ml). The resulting precipitate, collected by centrifugation was dissolved in *Tris*-HCl buffer 0.01 *M* pH 7.4 and dialyzed against the same buffer. Aliquots of enzyme solution were incubated for 20 min at 38 °C with AICR, 10-CHO-H_4 folate, KCl and *Tris*-HCl buffer[11]. The amount of AICR not removed was determined using the BRATTON and MARSHALL[8] method.

Results and discussion. The FIGlu excretion (Table I) results markedly increased in riboflavin-deficient rats as compared with controls ($P < 0.01$). Small but significant differences were observed in AIC excretion ($P < 0.05$). On the contrary, no difference was found in the liver levels of FIGlu transferase and AICR transformylase between riboflavin-deficient rats and control rats (Table II).

These data show that in riboflavin deficiency there is a failure to metabolize FIGlu. Since this cannot be ascribed to a decrease of FIGlu transferase, the decreased degradation of FIGlu might be the consequence of a lower storage of H_4 folate which is involved in the first step of FIGlu metabolism. In fact, when the enzyme preparation from livers of riboflavin-deficient rats was incubated with excess of H_4 folate, the amount of FIGlu metabolized was similar to that metabolized by control rats. Also AIC

Table I. Urinary excretion of FIGlu and AIC by riboflavin-deficient rats

Group	Animals in experiment	FIGlu excreted (nmoles/day per 100 g body wt.)	AIC excreted (nmoles/day per 100 g body wt.)
1	Control	498 ± 92 (6)[a]	71 ± 3.4 (6)
2	Riboflavin-deficient	2120 ± 113 (8) ($P < 0.001$)	88 ± 5.6 (6) ($P < 0.05$)

[a] Mean ± S.E.M.; No. of animals in parentheses; *P*, probability of difference (Student's *t*-test).

Table II. Liver FIGlu transferase and AICR transformylase in riboflavin-deficient rats

Group	Animals in experiment	FIGlu transferase[a] Units/g tissue	AICR transformylase[b] Units/g tissue
1	Control	34.2 ± 3.1 (6)[c]	14.7 ± 2.6 (6)
2	Riboflavin-deficient	36.3 ± 2.7 (6) (n.s.)	13.1 ± 2.1 (6) (n.s.)

[a] A unit of activity is defined as the amount of enzyme catalyzing the conversion of 0.05 μmole of FIGlu in 30 min. [b] A unit of activity is defined as the amount of enzyme catalyzing the removal of 0.1 μmole AICR in 20 min. [c] Mean ± S.E.M.; No. of animals in parentheses; n.s., not significant.

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metabolism is partially modified, but this cannot be ascribed to a decrease of AICR transformylase.

These results seem to demonstrate that riboflavin deficiency determines a decrease of storage of folate coenzymes. This could be considered the common biochemical lesion responsible in teratogenicity observed either in riboflavin or in folate deficiency.

Résumé. Des rats carencés en riboflavine, comparés aux contrôles, ont présenté une remarquable augmentation de la quantité de FIGlu éliminé. Une augmentation moins évidente a été observée dans l'élimination de AIC. Aucune variation, au contraire, en ce qui concerne les enzymes FIGlu transferase et AICR transformylase.

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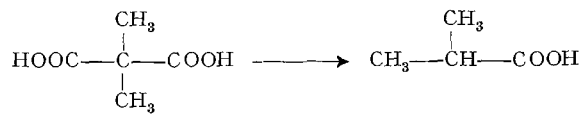
Microbial Degradation of Aliphatic Branched Compounds: Isobutyric, 2,2-Dimethylmalonic and 2,2-Dimethylsuccinic Acids[1]

In order to improve research on the microbial degradation of synthetic chemicals in soil, we have been trying to isolate microorganisms able to grow on aliphatic branched compounds supplied as only carbon source. These compounds are very resistant to bacterial oxidation, especially those having a quaternary carbon atom[2]. Concerning this, only KERSTIN[3] reports the isolation of microorganisms capable of growing on 2,2,4-dimethylpentane and MOHANRAO and MCKINNEY[4] the degradation of some quaternary carbon acids by activated sludges.

Results. The enrichment cultures were effected by direct incubation of river waters, added with the mineral salts of TAUSSON medium[5] and the required carbon source. At present, 3 microorganisms able to oxidize isobutyric, 2,2-dimethylmalonic and 2,2-dimethylsuccinic acids have been isolated.

From enrichments with isobutyric acid a motile, Gram-negative short rod, which produces a fluorescent green-yellow pigment on King B medium, but not phenazine pigment on King A medium[6], has been isolated. It does not produce gelatinase, and, by the criterion proposed by STANIER et al.[7], we may consider this organism to be a strain of *Pseudomonas putida*. This microorganism is unable to grow on 2,2-dimethylmalonic and 2,2-dimethylsuccinic acids.

After a long incubation time from an enrichment culture with 2,2-dimethylmalonic acid, a motile, Gram-negative short rod has been isolated which does not produce fluorescent and phenazine pigment, gelatinase and gas from carbohydrates; it may be tentatively assigned to the genus *Achromobacter*. First the growth on 2,2-dimethylmalonic acid was slight but very abundant after several subcultures. This strain is able to grow also on isobutyric acid as only carbon source, not on 2,2-dimethylsuccinic acid. Different items prove that the growth on 2,2-dimethylmalonic acid is really supported by this compound and not by isobutyric acid, which can be formed by non-enzymatic decarboxylation from 2,2-dimethylmalonic acid: 2,2-dimethylmalonic acid, whose purity had been tested after vacuum sublimation at 100 °C, was always supplied to the sterile media by amicrobial filtration. *Pseudomonas putida* isolated from isobutyrate enrichment cultures was unable to grow on 2,2-dimethylmalonic acid; washed cells of *Achromobacter sp.* grown on asparagine were not sequentially induced to oxidize 2,2-dimethylmalonic and isobutyric acids, while cells grown on 2,2-dimethylmalonic acid were sequentially induced to oxidize 2,2-dimethylmalonic and isobutyric acids; 2,2-dimethylmalonic acid was oxidized with the production of 2 moles of CO_2 per mole of substrate, while isobutyric acid with the production of 1 mole of CO_2 per mole of substrate. The same cells, heated at 100 °C for 2 min, do not give oxygen uptake and CO_2 release with the above compounds. From this it can be assumed that the first step of the degradation of 2,2-dimethylmalonic acid is the formation of isobutyric acid by enzymatic decarboxylation.



2,2-dimethylmalonic acid — isobutyric acid

From enrichment cultures with 2,2-dimethylsuccinic acid a motile, Gram-negative short rod has been isolated, different from the above strain, which does not produce fluorescent and phenazine pigment, gelatinase and gas from carbohydrates: it may be tentatively adscribed to the genus *Achromobacter*. This microorganism is able to grow on 2,2-dimethylsuccinic, 2,2-dimethylmalonic and isobutyric acids. Washed cells of this strain grown on 2,2-dimethylsuccinic acid immediately oxidize 2,2-dimethylsuccinic, not 2,2-dimethylmalonic acid. Research is in progress to isolate microorganisms able to grow on hydrocarbons with quaternary carbon atom.

Riassunto. Da colture di arricchimento si è isolato un ceppo di *Pseudomonas putida* capace di crescere in presenza di acido isobutirrico, un *Achromobacter sp.* capace di crescere in presenza degli acidi 2,2-dimetilmalonico e isobutirrico e un altro *Achromobacter sp.* capace di crescere in presenza degli acidi 2,2-dimetilsuccinico, 2,2-dimetilmalonico ed isobutirrico. Esperienze di induzione sequenziale hanno dimostrato che l'acido 2,2-dimetilmalonico viene degradato ad acido isobutirrico per decarbossilazione enzimatica.

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