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ACTIVITY AND DISTRIBUTION OF THE ENZYMES OF URIDYLATE SYNTHESIS FROM OROTATE IN ANIMAL TISSUES

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SUMMARY

1. Activity and distribution of orotidine 5'-monophosphate pyrophosphorylase (EC 2.4.2.10) and orotidine 5'-monophosphate decarboxylase (EC 4.1.1.23), were studied. The activities of both enzymes catalyzing uridylate biosynthesis from orotate were measured by the formation of $^{14}CO_2$ from [*carboxy*-^{14}C]orotate and orotidine 5'-monophosphate (OMP), respectively. Improved conditions for their determination are given, including the use of excess EDTA, for the assay of OMP pyrophosphorylase with endogeneous OMP decarboxylase as indicator enzyme.

2. The subcellular distribution of both enzymes in rat liver indicates an exclusive localization in the cytosol.

3. The specific activity of OMP pyrophosphorylase in different rat tissues was found to decrease in the following order: regenerating liver > spleen > liver > gut > kidney > brain > skeletal muscle. The order of OMP decarboxylase activity was: spleen > regenerating liver > liver > kidney > gut > brain > skeletal muscle.

4. The hepatic activities of the two enzymes were decreasing during the postnatal development.

5. In the livers of different animal species the specific activities of both enzymes were highest in mice, lowest in guinea pigs, and intermediate in chicken and rats.

6. Under a variety of conditions, including fast growing tissues, OMP pyrophosphorylase and OMP decarboxylase showed closely parallel, possibly coordinate changes in activity.

INTRODUCTION

Uridine phosphates serve as essential precursors in the biosynthesis of nucleic acids and UDP-sugars; the latter function as glycosyl donors for saccharide, glycoprotein, and glycolipid synthesis[3,4]. Regulatory properties and a possible limiting role in the pyrimidine biosynthetic pathway of the enzymes catalyzing the conversion of

Abbreviations[1,2]: OMP, orotidine 5'-monophosphate; PRPP, phosphorylribose 1-pyrophosphate.

orotate to uridylate were reported[5–12]. Simultaneous deficiency of both enzymes is observed in hereditary orotic aciduria[9,10,13,14]. Increased activities of these enzymes were found in fast growing tissues, *e.g.* regenerating and neonatal liver[11,15,16].

Evidence was presented by BRESNICK[11] indicating that OMP pyrophosphorylase (orotidine 5′-phosphate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.10) is rate limiting after partial hepatectomy. OMP decarboxylase (orotidine 5′-phosphate carboxylyase, EC 4.1.1.23) was shown to be competitively inhibited by uridylate[6,7,17] and 6-azauridine 5′-monophosphate[5,7,9].

In this paper improved radiochemical assays for OMP pyrophosphorylase and OMP decarboxylase are described. The subcellular distribution in rat liver and the activity of both enzymes in various tissues and animal species are communicated.

MATERIALS AND METHODS

Phosphorylribose 1-pyrophosphate (PRPP) tetrasodium salt was obtained from Sigma Chemical Co. (St. Louis, Mo.), choline orotate from Dr. Falk, REMEFA, (Freiburg), orotidine 5′-monophosphate trilithium salt from Calbiochem (Los Angeles), Triton X-100 from SERVA GmbH (Heidelberg).

[*carboxy*-^{14}C]Orotic acid hydrate (10.2 mC/mmole) and [*carboxy*-^{14}C]orotidine 5′-monophosphate triammonium salt (21.0 mC/mmole) were purchased from New England Nuclear Corp. (Boston). All other reagents used were analytical grade.

Animals

Female Wistar rats (Ivanovas, Kisslegg, Germany) were fed an unrestricted commercial diet (Altromin, from Altromin GmbH, Lage/Lippe). Male Ha/ICR (Swiss)-mice (SPF) supplied by MUS–Rattus AG, (Brunnthal, Germany) were fed the same diet as the rats. White hens (Leghorn) were purchased from local breeders. Female guinea pigs BFA, ZH Kisslegg were obtained from Ivanovas (Kisslegg, Germany). Fed animals were used throughout, tissues were removed immediately after decapitation between 8 and 10 a.m.

Protein assays

Protein was determined by the biuret method[18]. Comparative measurements were made by the methods of LOWRY[19] and WARBURG AND CHRISTIAN[20]. In tissues which could not easily be freed of blood by perfusion, the protein content was corrected for blood protein. This was done by hemoglobin determination[21] in hemolyzed tissue homogenates, 1.5 g hemoglobin corresponding to 2.0 g of blood protein. The enzyme activities under investigation were extremely low in the blood. Enzyme activities are given at 25° in international units (1 I.U. = 1 μmole/min).

Enzyme preparation

OMP decarboxylase from baker's yeast (Hefefabrik Weingarten) was partially purified according to HEPPEL AND HILMOE[22]. In some experiments a lyophilized preparation from yeast, kindly supplied by Boehringer Mannheim GmbH, was used.

Preparation of supernatant fractions of tissues

Livers were perfused *in situ* with 20 ml of cold 0.25 M sucrose, excised, and

weighed immediately. After addition of 3 vol. of cold 0.25 M sucrose the tissue was homogenized with a glass–teflon apparatus described by POTTER AND ELVEHJEM[23] (500 rev./min, 10 strokes), and centrifuged at 0° for 1 h at 105 000 × *g* in a Spinco L 50 centrifuge. The supernatant fraction could be stored at 5° without detectable loss of activity for at least 30 h. The dilution of the supernatant of 1 g of tissue by 3 ml 0.25 M sucrose was calculated to be 4-fold in order to express the enzyme activities as mU/g wet weight. Tissue samples from kidney, spleen, brain, skeletal muscle, and gut were not perfused. Before homogenizing the gut was rinsed three times with 0.25 M sucrose and cut into small pieces. Skeletal muscle was cut with a micro-mincer as described by PETTE[24]. Further preparation of the supernatants was performed in the same way as for liver tissue.

Cell fractionation

Livers were excised after perfusion *in situ* with cold 0.25 M sucrose. The sequential extraction procedure was performed as described by PETTE[24] with the following modifications: All extraction steps were carried out with 0.25 M sucrose. Fraction S4 was prepared by stirring thoroughly the residue of Step 3 in 0.25 M sucrose. To all fractions Triton X-100 in a final concentration of 0.1% was added. Lactate dehydrogenase (EC 1.1.1.27) for the cytosol and glutamate dehydrogenase (EC 1.4.1.3) for the particulate fraction were used as marker enzymes; they were assayed according to BERGMEYER *et al.*[25] and SCHMIDT[26], respectively.

Incubation vessels and measurement of $^{14}CO_2$

Construction and assembly of incubation vessels were similar to those described by WOHLHUETER AND HARPER[27]. $^{14}CO_2$ produced by decarboxylation of labeled OMP was trapped in 0.2 ml of 1 M KOH (ref. 28) after injection of 0.9 M $HClO_4$ into the incubation mixture. The scintillation vials were separated from the test tubes and 10 ml of scintillator solution as described by BRAY[29], containing 4% (w/v) silica powder, were added. The counting efficiency was 92% using a Packard Tricarb Scintillation Spectrometer Model 3380.

OMP pyrophosphorylase activity

Assay I

50 μl of high-speed supernatant were added to 20 μl yeast OMP decarboxylase (20 mg protein per ml, specific activity 8.1 mU/mg protein; 16.7 mg lyophilized extract of yeast suspended in 0.5 ml 50% glycerol containing 4 mM reduced glutathione; the latter does not affect OMP decarboxylase activity[25]). The reaction was initiated by addition of 100 μl of a mixture containing 4 mM PRPP, 13 mM EDTA (EDTA stabilizes PRPP in the stock solution[15]; the excess of Mg^{2+} in the reaction mixture over EDTA was 7.1 mM), 230 mM Tris–HCl buffer (pH 8.0), 20 mM $MgCl_2$, 1 mM [*carboxy*-^{14}C]orotate ($1.1 \cdot 10^5$ disint./min). After 10 min of incubation at 25° the reaction was discontinued by injection of 0.5 ml 0.9 M $HClO_4$. The test tubes were shaken for 1 h at 30° to allow the quantitative absorption of $^{14}CO_2$ by KOH in the scintillation vial. Assay I, based on the work of LIEBERMAN *et al.*[8], was used in most of the experiments.

Assay II

50 μl of high-speed supernatant were mixed with 100 μl of the reaction mixture

described above. After 10 min incubation at 25° the reaction was terminated by injection of 50 μl 0.25 M EDTA (adjusted to pH 8.0 with NaOH) through the serum bottle caps. At this pH EDTA removes the free Mg^{2+} required for OMP pyrophosphorylase activity. For complete decarboxylation of ^{14}C-labeled OMP by endogeneous or added OMP decarboxylase the samples were incubated at about 35° for at least 1 h. The following steps of the experimental procedure including injection of 0.5 ml 0.9 M $HClO_4$ were performed as described for OMP pyrophosphorylase Assay I. The activities in rat liver measured with Assay II were about 10% higher than those obtained with Assay I.

Assay of OMP decarboxylase activity

50 μl of high-speed supernatant were incubated for 10 min at 25° with 100 μl reaction mixture consisting of 170 mM Tris maleate (pH 6.25), 7 mM EDTA and 0.34 mM [*carboxy*-^{14}C]OMP ($3 \cdot 10^4$ disint./min). The reaction was discontinued by injection of 0.5 ml 0.9 M $HClO_4$. All further steps were carried out as described in OMP pyrophosphorylase Assay I.

RESULTS AND DISCUSSION

Activity and subcellular distribution of OMP pyrophosphorylase

Under optimal conditions (pH 8; PRPP, >2 mM) the production of $^{14}CO_2$ from [*carboxy*-^{14}C]orotate was linear with time for at least 12 min, and strictly linear with protein concentrations up to 8 mg/ml in both assays using liver supernatant with a specific activity of 0.5 mU/mg. Assay II, which makes use of the different requirement for divalent cations of OMP pyrophosphorylase and OMP decarboxylase, is advantageous as the addition of partially purified OMP decarboxylase can be avoided. The ratio of activities at 25° and 37° was 1:2.15.

The subcellular distribution of OMP pyrophosphorylase activity in liver was

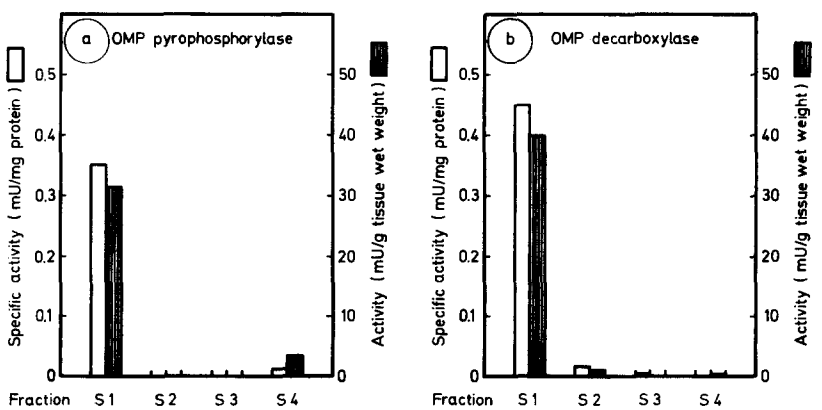

Fig. 1. Subcellular distribution of OMP pyrophosphorylase (a) and OMP decarboxylase (b) in rat liver. The liver homogenate was extracted sequentially by the procedure of PETTE[24]; S1 represents the first supernatant (105 000 × *g*, 20 min), S2 and S3 the supernatants of the subsequent washings, S4 the 4 times washed particulate fraction. The S1 fraction contained <1% of glutamate dehydrogenase activity, whereas the particulate fraction (S4) was sufficiently free of contaminating lactate dehydrogenase activity (<10%).

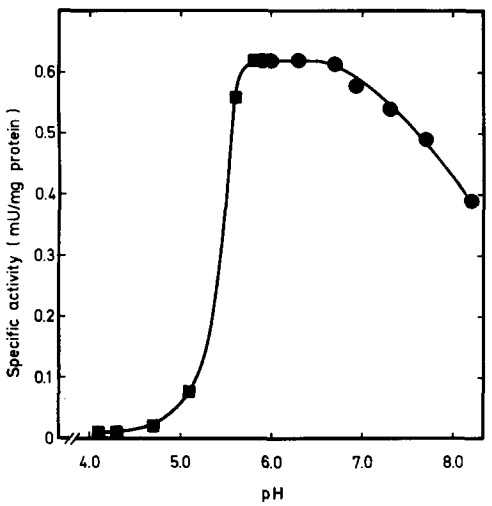

Fig. 2. Effect of pH on the activity of OMP decarboxylase in the high-speed supernatant fraction from rat liver. Reaction rates were measured as $^{14}CO_2$ liberated by decarboxylation of [*carboxy*-^{14}C] OMP during 10 min incubation at 25°. 0.18 M acetate buffer was used for pH values from 4.1 to 5.8; from 5.9 to 8.2 0.05 M Tris maleate buffer. The experimental procedure was performed as described in MATERIALS AND METHODS.

studied after cell fractionation according to the method of PETTE[24]. Fig. 1a indicates that OMP pyrophosphorylase is localized almost exclusively in the cytosol. A corresponding conclusion was reached by BAER AND LANG[16] who employed less specific methods. Inhibition of OMP pyrophosphorylase by 5-fluoroorotate was described[30,31]. In the present assay 0.5 mM 5-fluoroorotate caused a 90% inhibition. Strong inhibition of the enzyme by ammonium sulfate at concentrations above 0.1 M was observed.

Activity and subcellular distribution of OMP decarboxylase

The full activity of the enzyme in the presence of excess EDTA[7] allows the measurement of OMP decarboxylase activity without interference of Mg^{2+}-dependent reactions including OMP pyrophosphorylase and OMP-splitting phosphohydrolases.

Simultaneous determinations of UMP formed from [*carboxy*-^{14}C]OMP by an enzymatic assay[32] and of $^{14}CO_2$ were in good agreement confirming the reliability of the radioassay. The pH optimum for rat liver OMP decarboxylase in the high-speed supernatant was determined to be between pH 5.9–6.5 (Fig. 2) as compared to pH 7.18 described by BLAIR AND POTTER[6] and 7.4 determined for the purified rat liver enzyme[7].

The ratio of activities at 25° and 37° was 1:2.4. The decarboxylation of OMP in the assay described above was linear with time for at least 12 min and with protein concentration (up to 12 mg/ml).

Analysis of the subcellular distribution of OMP decarboxylase showed that the activity was localized entirely in the cytosol (Fig. 1b) corresponding to the localization of OMP pyrophosphorylase (Fig. 1a).

6-Aza-UMP has been shown to function as a most effective inhibitor of OMP decarboxylase from various sources both *in vitro* and *in vivo*[5,7,9,10,31,33,34]. In the assay described, 1 mM 6-aza-UMP completely inhibited the enzyme. Inhibition of liver OMP decarboxylase by UMP[6,7] does not seem to be of physiological significance as

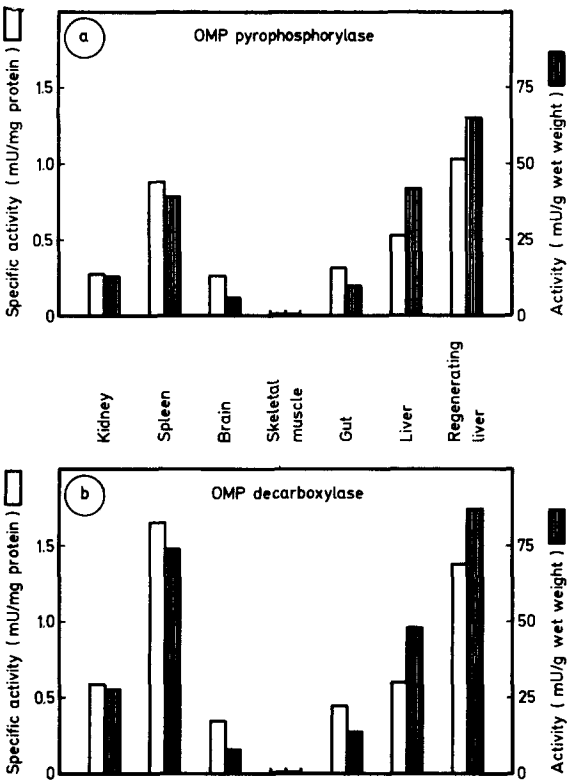

Fig. 3. Distribution of OMP pyrophosphorylase (a) and OMP decarboxylase (b) activity in various tissues of female rats (150 g, 60–80 days of age). Regenerating liver was analyzed 5 days after removal of 70% of the hepatic tissue. Mean values from at least two animals. For further details see MATERIALS AND METHODS.

TABLE I

AGE DEPENDENCY OF THE SPECIFIC ACTIVITIES OF OMP PYROPHOSPHORYLASE AND OMP DECARBOXYLASE IN THE HIGH-SPEED SUPERNATANT FRACTION OF RAT LIVER

Enzyme assays were carried out as described in MATERIALS AND METHODS. Mean values are given as mU/mg protein ± S.D. The hepatic protein content (mg/g wet wt.) at 1, 10, and 21 days after birth was 43%, 59%, and 92% that of adult rats, respectively.

Time after birth (days)	*Body weight (g)*	*OMP pyrophosphorylase (mU/mg protein)*	*OMP decarboxylase (mU/mg protein)*
1	8	2.04 ± 0.18 (4)	2.21 ± 0.58 (4)
6	15	1.48 ± 0.15 (4)	1.71 ± 0.23 (4)
10	20	1.01 ± 0.44 (4)	1.50 ± 0.09 (4)
21	40	1.32 ± 0.06 (4)	1.47 ± 0.08 (4)
60–80	150	0.53 ± 0.04 (12)	0.60 ± 0.09 (12)
230–250	270	0.48 ± 0.01 (4)	0.67 ± 0.05 (4)

1 mM UMP was without effect and as the hepatic UMP concentration is in the range of 0.05 mM (ref. 32). A 10% inhibition of the enzyme was measured with 1 mM UDP-glucose.

Activities in different rat tissues

The activities of OMP pyrophosphorylase and OMP decarboxylase were determined in various rat tissues (Fig. 3). The low activity in muscle was also observed in the uncentrifuged homogenate of this tissue.

Both enzymes showed a coordinate activity. Although the specific activities in various tissues differed by a factor of more than four, the ratio of OMP decarboxylase and OMP pyrophosphorylase remained in the range of 1 to 2 with a mean of 1.4

TABLE II

HEPATIC OMP-PYROPHOSPHORYLASE ACTIVITIES IN SEVERAL SPECIES

Animals and experimental procedures including the preparation of high-speed supernatants are described in MATERIALS AND METHODS. Mean values ± S.D. are given.

Animal species	*Body weight (g)*	*Specific activity (mU/mg protein)*	*Activity (mU/g wet wt.)*
Mice	15	0.92 ± 0.11 (6)	56.5 ± 7.5 (6)
Mice	35	0.62 ± 0.05 (3)	36.4 ± 1.1 (3)
Chicken	1800	0.42 ± 0.04 (3)	25.1 ± 1.8 (3)
Guinea pigs	200	0.05 ± 0.04 (3)	4.1 ± 2.7 (3)
Rats	150	0.53 ± 0.04 (12)	41.9 ± 5.9 (12)

(Fig. 3). Distribution patterns in various rat tissues of other enzymes of pyrimidine biosynthesis[34,35] differ markedly from that described above.

Age-dependent changes of hepatic activities

The specific activities of OMP pyrophosphorylase and OMP decarboxylase were assayed in livers of rats at different age. Rats show a decrease of both enzyme activities during postnatal development (Table I).

The specific activity (mU/mg liver protein) of several enzymes of pyrimidine biosynthesis is correlated with growth velocity in fetal, neonatal[16,34,36–39], and regenerating liver[11,15,38], and in several tumor cells[15,34,36–38]. These growth-dependent changes of the activities of OMP pyrophosphorylase and OMP decarboxylase measured *in vitro* under optimal conditions (Table I) are correlated to the respective rates of stimulated uridylate synthesis *in vivo*[40] although their capacity *in vitro* (Table I, Fig. 3) exceeds the estimated rate *in vivo* several fold. The latter was studied after depletion of the hepatic uridine phosphate and UDP-hexose pools and measured as the increase of the sum of uracil nucleotides with time[40]. A value of 5.8 nmoles · min^{-1} · g wet $wt.^{-1}$ can be regarded as the minimum rate of stimulated uridylate synthesis in adult rat liver[40]. This value was increased in neonatal rat liver (10 days after birth) by a factor 2.3, and 5 days after partial hepatectomy by a factor 1.7; the corresponding increase of specific OMP pyrophosphorylase activity was 1.9- and 1.9-fold, that of OMP decarboxylase 2.5- and 2.3-fold, respectively.

TABLE III

HEPATIC OMP DECARBOXYLASE ACTIVITIES IN SEVERAL SPECIES

Animals and experimental procedures are described under MATERIALS AND METHODS. Mean values ± S.D. are given.

Animal species	*Body weight (g)*	*Specific activity (mU/mg protein)*	*Activity (mU/g wet wt.)*
Mice	15	1.58 ± 0.06 (6)	96.6 ± 6.7 (6)
Mice	35	0.88 ± 0.06 (3)	52.2 ± 2.2 (3)
Chicken	1800	0.67 ± 0.07 (3)	40.2 ± 4.4 (3)
Guinea pigs	200	0.11 ± 0.03 (3)	8.5 ± 1.3 (3)
Rats	150	0.60 ± 0.09 (12)	48.1 ± 12.1 (12)

Activities in different animal species

The hepatic activities of OMP pyrophosphorylase and OMP decarboxylase in rats, mice, chicken, and guinea pigs differed over a wide range (Tables II and III). The correlation between enzyme activities and age in mice was analogous to that observed with rats. In chicken brain OMP pyrophosphorylase activity was 0.21 ±0.03 (S.D.) mU/mg protein, OMP decarboxylase activity 0.29 ± 0.06 (S.D.) mU/mg protein ($n = 6$). A possible limiting role of one or both of the enzymes studied in the *de novo* uracil nucleotide biosynthesis in animal tissues is a subject of further investigations.

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