Methionine metabolism in isolated perfused livers from rats fed on zinc-deficient and restricted diets*

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(Received 7 November 1985 – Accepted 8 April 1986)

1. Sulphur amino acid metabolism in livers from rats which had been fed ad lib. on a restricted diet (malnourished) or a Zn-deficient diet was investigated. Perfusion of normal livers with different amounts of L-methionine revealed that homocysteine was recycled four to five times before being eliminated via the transsulphuration pathway.

2. From the perfusion experiments, we found no evidence that any of the enzymes involved in recycling homocysteine back to methionine (methylation pathway) were adversely effected by Zn deficiency or malnutrition.

3. The intracellular concentration of cystathionine and S-adenosylmethionine increased in all livers in response to increased concentrations of L-methionine (L-Met) in the perfusate.

4. The intracellular concentration of S-adenosylhomocysteine remained the same in all livers regardless of the concentration of perfusate methionine.

5. Although homocysteine did not accumulate in the cell, it was excreted into the perfusate. The amount of homocysteine in the perfusate of livers from Zn-deficient rats was higher than either ad lib. or pair-fed rats.

6. The breakdown of homocysteine, via the transsulphuration pathway, was augmented by Zn deficiency. This was apparent from the greater amount of α-ketobutyrate excreted by livers from Zn-deficient rats compared with pair-fed or ad lib.-fed controls.

7. The increase in metabolism of L-Met, via the transsulphuration pathway, in the livers from Zn-deficient rats appears to reflect the lack of demand for this compound in protein synthesis and methylation reactions (Wallwork & Duerre, 1985).

L-Methionine (L-Met) is an essential amino acid required for most cellular processes. L-Met is utilized in the cell for protein synthesis and as the precursor of S-adenosylmethionine (Fig. 1). In more than 100 reactions S-adenosylmethionine (Ado-Met) functions as the methyl donor and in others it is the donor of propylamino and α-aminobutyryl side chains. S-Adenosylhomocysteine (Ado-Hcy), one of the products of transmethylation reactions involving Ado-Met, can act as a competitive inhibitor of most, if not all, of these reactions (Zappia et al. 1969; Deguchi & Barchas, 1971; Coward et al. 1972; Finkelstein et al. 1974; Hoffman et al. 1980).

The primary mechanism for the breakdown of Ado-Hcy in eucaryotic cells is via the reversible S-adenosylhomocysteinase (EC 3.3.1.1), to yield adenosine and L-homocysteine (de la Haba & Cantoni, 1959; Walker & Duerre, 1975; Schatz et al. 1979). In the presence of S-methyltetrahydrofolate, the Hcy formed is methylated to regenerate L-Met. Thus, the carbon skeleton of L-Met is recycled several times before being eliminated via cystathionine.

* This investigation was supported in part by the United States Department of Agriculture Cooperative Agreement no. 12-14-3001-294.
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or incorporated into proteins (Mudd & Poole, 1975; Hoffman et al. 1980). The high demand for methyl groups and rapidity with which the C skeleton is recycled can be readily demonstrated by perfusing rat livers with t-Met radiolabelled in the methyl and carboxyl positions (Hoffman et al. 1980; Wallwork & Duerre, 1985; Wallwork et al. 1986).

When livers from malnourished or ad lib.-fed animals were perfused with a limited amount of t-Met radiolabelled in the methyl and carboxyl positions, the methyl group was found to turn over four to five times faster than the C skeleton (Wallwork & Duerre, 1985; Wallwork et al. 1986). In contrast, the methyl group of t-Met turned over at a much slower rate in livers from zinc-deficient rats. This relatively slow turnover in methyl groups was most likely due to the availability of methyl acceptors (Wallwork & Duerre, 1985).

**MATERIALS AND METHODS**

**Chemicals**

L-[Methyl-3H]Met (26 Ci/mmol) and L-[carboxyl-14C]Met (51 Ci/mmol) were purchased from New England Nuclear, Boston, MA and Amersham Corp., Arlington Heights, IL respectively. L-Met and other essential amino acids were obtained from Sigma Chemical Co., St Louis, MO.

**Animals**

Long–Evans male rats (114–125 g) were maintained individually in suspended stainless-steel cages with precautions to minimize trace-element contamination (Wallwork et al. 1981). Animals were kept on a 12 h light–12 h dark cycle and were fed daily at 08:30 hours. The diet contained 200 g egg-white/kg, was fortified with added biotin and contained < 1 μg
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Zn/kg. The composition of the diet has been published previously (McKenzie et al. 1975) except that chlorotetracycline hydrochloride was omitted and 1·0 g inositol/kg was added (Teklad Mills, Madison, WI). One group of rats was fed on this diet and given distilled, deionized water (Zn-deficient). Because of the anorexia associated with Zn deficiency, two types of Zn-supplemented control rats were used. On an individual basis, one group (pair-fed) was fed the same amount of diet consumed by the Zn-deficient animals on the previous day. The other group of rats (ad lib.-fed) was given the diet ad lib. Zn-supplemented controls were given distilled, deionized water containing 25 μg Zn (as zinc acetate)/kg. The dietary regimen lasted for 21–23 d.

Technique of liver perfusion

Livers were isolated from the rats under light anaesthesia with diethyl ether, following cannulation of the bile duct, portal vein and thoracic inferior vena cava, in that order. Heparin was injected into the abdominal inferior vena cava before interruption of circulation. The apparatus used for liver perfusion was as described by Veneziale et al. (1967). Isolated livers were perfused for 20 min by recycling 110 ml perfusate, pH 7·45, consisting of rat erythrocytes suspended in Krebs–Ringer bicarbonate buffer (Cohen, 1959), containing 30 g bovine serum albumin/1 and 150 mg glucose/ml. The perfusate was oxygenated with oxygen:carbon dioxide (95:5, v/v). The erythrocytes were from 75 ml rat blood obtained by cardiac puncture. The blood was mixed with 500 units heparin and erythrocytes sedimented by centrifugation at 120 g for 30 min at 4°, followed by two washes with physiological saline (9 g sodium chloride/l). Erythrocytes, for the perfusion of livers from Zn-deficient rats, were obtained from animals fed on a similar diet.

Following the 20 min equilibration period, all the essential amino acids except L-Met were added at twice plasma levels (0·2 mm-arginine, 0·12 mm-histidine, 0·16 mm-isoleucine, 0·24 mm-leucine, 0·9 mm-lysine, 0·1 mm-phenylalanine, 1·2 mm-threonine, 0·22 mm-tyrosine, 0·34 mm-valine (Jacob & Crandall, 1972)). Varying amounts of L-Met, containing L-[methyL-P]Met and L-[carboxy-P4C]Met, were added at the same time. Livers were perfused by recycling the perfusate at a rate of 17 ml/min. Samples of the perfusate were taken every 1 min for the determination of the uptake of L-Met and measurement of Hcy, cystathionine and α-ketobutyrate. At 20-min intervals, liver biopsy samples (0·5–1·5 g) were taken, frozen in liquid nitrogen and stored at −80° until analysed for Hcy, cystathionine and α-ketobutyrate.

Criteria for viable livers were (a) macroscopic appearance, (b) production of bile, (c) rate of gluconeogenesis and (d) rate of uptake of radioactive L-Met.

Chromatography of sulphur-containing amino acids and α-ketobutyrate

L-Met, Hcy, cystathionine and α-ketobutyrate were measured in liver biopsy and perfusate samples taken at designated time points during the perfusion. Liver samples (0·3–1·0 g) were homogenized in 2 ml trichloroacetic acid (50 g/l), followed by centrifugation and filtration through 1 μm porosity, glass-fibre filters. The extraction procedure was repeated and the filtrates pooled. Perfusate samples (5–10 ml) were deproteinized with trichloroacetic acid (50 g/l) and treated in the same way. Amino acids and α-ketobutyrate were fractionated on cation-exchange resin employing a Technicon automatic amino acid analyzer (Duerre, 1968). The column effluent was split with a stream divider. Half the effluent passed through the amino acid analyzer while the remaining effluent was used for the determination of radioactivity by channels ratio technique using a Packard scintillation spectrometer. The compounds, L-homocysteine and cystathionine, were at or below the assayable limit of the ninhydrin reaction. These compounds were quantified by summing the radioactivity (14C) under the peak and dividing by the specific radioactivity of the substrate, L-Met.
Statistical analysis

Group differences were evaluated by one-way analysis of variance and the differences between individual means were assessed for significance using Scheffé contrasts (Scheffé, 1959). Values are given as means with their standard errors.

Table 1. Intracellular concentration of sulphur-containing compounds (nmol/g fresh weight) in isolated livers after perfusion with 60 μM-L-methionine for 30 or 60 min

(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Period of perfusion and dietary regimen</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
<th>L-Methionine</th>
<th>Ado-Met</th>
<th>Ado-Hey</th>
<th>Cystathionine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>30 min:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad lib.-fed</td>
<td>6</td>
<td>269 a</td>
<td>12</td>
<td>9-7 a</td>
<td>1-2</td>
<td>91 a</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>4</td>
<td>169 b</td>
<td>10</td>
<td>6-0 b</td>
<td>0-6</td>
<td>80 b</td>
</tr>
<tr>
<td>Zn-deficient</td>
<td>6</td>
<td>163 b</td>
<td>8</td>
<td>5-9 b</td>
<td>0-9</td>
<td>101 b</td>
</tr>
<tr>
<td>60 min:</td>
<td></td>
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<td></td>
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<tr>
<td>Ad lib.-fed</td>
<td>6</td>
<td>269 a</td>
<td>12</td>
<td>9-7 a</td>
<td>1-2</td>
<td>90 a</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>4</td>
<td>169 b</td>
<td>10</td>
<td>6-0 b</td>
<td>0-6</td>
<td>82 b</td>
</tr>
<tr>
<td>Zn-deficient</td>
<td>6</td>
<td>163 b</td>
<td>8</td>
<td>5-9 b</td>
<td>0-9</td>
<td>113 b</td>
</tr>
</tbody>
</table>

Ado-Met, S-adenosylmethionine; Ado-Hey, S-adenosylhomocysteine; Hey, homocysteine.

There was no significant difference in the level of L-methionine, Ado-Met, Ado-Hey, cystathionine and Hey in livers taken from either the top or bottom of the feeding cycle in Zn-deficient or pair-fed rats. Consequently, values from within these groups were pooled.

a,b Values within a column with unlike superscript letters were significantly different (analysis of variance and Scheffé contrasts): P < 0-05.

α-Ketobutyrate passed through the column near the void volume. This compound was quantified by the 2,4-dinitrophenylhydrazine reaction (Friedemann & Haugen, 1943) and by summing the radioactivity (14C) under the peak. The identity of α-ketobutyrate under the peak was assessed by thin-layer chromatography of the 2,4-dinitrophenylhydrozone (Dancis et al. 1963). The 2,4-dinitrophenylhydrozone was dissolved in ethanol, spotted on a Silica gel plate and developed with isoamyl alcohol:0·25 M-ammonium hydroxide (20:1, v/v). About 90% of the radioactivity under the peak co-chromatographed with an authentic sample of the 2,4-dinitrophenylhydrozone of α-ketobutyrate. The radioisotope assay was much more sensitive than the 2,4-dinitrophenylhydrazine reaction; therefore, the results are given as the sum of the radioactivity (14C) under the peak divided by the specific radioactivity of the substrate, L-Met.

Chromatography of Ado-Met and Ado-Hcy

Liver samples (0·3-1·5 g) were homogenized in two volumes of sulphosalicylic acid (50 g/l), followed by centrifugation and filtration through 1 μm porosity glass-fibre filter. The extraction procedure was repeated and the filtrates pooled. Ado-Met and Ado-Hcy were fractionated by high pressure liquid chromatography on Vydac cation resin (Hoffman et al. 1980). Elution of the compounds was followed at 251 nm. The amount of Ado-Met and Ado-Hcy under the peaks was quantified by comparing against known standards using an integrator. The column effluent was collected in 2 ml fractions for the determination of the amount of 3H and 14C under the peaks. Radioactivity was determined by channels ratio technique using a Packard scintillation spectrometer.

Statistical analysis

Group differences were evaluated by one-way analysis of variance and the differences between individual means were assessed for significance using Scheffé contrasts (Scheffé, 1959). Values are given as means with their standard errors.
**RESULTS**

Rats fed on a Zn-deficient diet displayed typical signs of Zn deficiency such as anorexia, cyclic feeding behaviour and retarded growth. Young rats became anorexic within 3–4 d of eating a Zn-deficient diet and subsequently ate in a cyclic manner, i.e. these animals consumed low amounts of diet for 1–2 d followed by 2 d of near-normal food intake. Depending on the length of the Zn-deficient regimen, the pair-fed control animals can be more- or less-severely influenced by the low dietary intake. In growing rats, pair feeding to Zn-deficient animals causes a slower growth rate than ad lib. feeding (Table 1); therefore, pair-feeding reflects the effects of malnutrition on the animal. The livers from these animals also do not respond to perfusion with adequate nutrients as do livers from normal ad lib.-fed animals. When livers from severely Zn-deficient rats were perfused with L-Met the compound was taken up at a rate equivalent to that by livers from ad lib.-fed controls (35 nmol/g tissue per min), but significantly slower than that by livers from their pair-fed mates (55 nmol/g tissue per min). These results are similar to those previously published (Wallwork & Duerre, 1985; Wallwork et al. 1986).

When livers from pair-fed rats were perfused with plasma levels of L-Met (60 µM), the intracellular concentration of L-Met was equivalent to that in livers from either the Zn-deficient or ad lib.-fed groups. However, the amount of Ado-Met remained at below normal concentrations (Table 1). This was not the result of impaired Ado-Met synthesis, since these livers synthesized as much or more Ado-Met as livers from either Zn-deficient or ad lib.-fed controls when L-Met was increased to 240 µM (Table 2). Apparently, the concentration of Ado-Met in the liver is quite readily altered by the size of the internal pool of L-Met. This is consistent with previously published results (Hoffman et al. 1980). Although the internal pool of Ado-Met fluctuated markedly, the intracellular concentration of Ado-Hcy remained constant regardless of the external or internal concentration of perfusate L-Met (Tables 1 and 2). Similarly, the Ado-Hcy concentration remained constant regardless of the period of time the animals were fed on a Zn-deficient diet.

When we compared livers from pair-fed rats that were at the bottom and top of the feeding cycle, there were no significant differences in the concentrations of L-Met, Ado-Hcy...
or Ado-Met in the liver. Similar results were obtained when we compared the concentrations of these compounds in livers from Zn-deficient rats at various parts of the feeding cycle (data not presented).

Ado-Hcy is normally degraded via S-adenosylhomocysteine to yield adenosine and Hcy (Fig. 1). Free or bound Hcy could not be detected in the tissue, irrespective of the amount of l-Met added to the perfusate (Tables 1 and 2). Conversely, Hcy was detectable in the perfusate. This compound increased in the perfusate with time reaching a level of 3·3 nmol/ml after 60 min when livers from Zn-deficient rats were perfused with 60 μM l-Met (Fig. 1). Although not statistically different, the amount of Hcy in the perfusate of livers from ad. lib.-fed rats was less (2·1 nmol/ml). Under these conditions Hcy could not be detected in the perfusate of livers from the pair-fed rats. Increasing the concentration of l-Met to 240 μM resulted in a concomitant rise in perfuse Hcy (Fig. 2(b)). Hcy reached a level of 4·0 nmol/ml and 6·2 nmol/ml after 60 min for livers from ad lib.-fed and Zn-deficient animals respectively. Furthermore, Hcy was detectable in the perfusate of livers from the pair-fed group, reaching a concentration of 2·0 nmol/ml after 60 min (Fig. 2(b)).

Hcy can be recycled back to l-Met via the enzyme betaine-homocysteine methyltransferase (EC 2.1.1.5) or 5-methyltetrahydrofolate homocysteine methyltransferase (EC 2.1.1.13). Hcy can also be converted to cystathionine via the enzyme cystathionine-β-synthase (EC 4.2.1.22) (Fig. 1). Cystathionine concentrations reached levels of
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13–19 nmol/g tissue in livers from all experimental groups within 30 min of perfusion with 60 μM-L-Met (Table 1). The concentration of cystathionine remained at this level throughout the duration of the experiment, except that the level declined in livers from the pair-fed animals. When the perfusate L-Met was increased to 240 μM, the cystathionine concentration increased to 27–36 nmol/g tissue within 30 min (Table 2). Again the level of cystathionine in the livers from pair-fed rats decreased with time.

Cystathionine also was excreted into the perfusate by the livers from all experimental groups (Fig. 3). In livers, which were perfused with 60 μM-L-Met, the concentration of cystathionine reached 3–5 nmol/ml perfusate within 20 min. When perfusate L-Met was increased to 240 μM, the concentration of cystathionine increased to 11–13 nmol/ml within 10 min. Under both sets of conditions, the cystathionine in the perfusate from pair-fed livers decreased significantly with time. In contrast, the cystathionine concentration remained relatively stable in the perfusate from livers from Zn-deficient or ad lib.-fed rats.

Cystathionine is degraded to cysteine and α-ketobutyrate via cystathionine γ-lyase (EC 4.4.1.1) (Fig. 1). The latter compound was readily excreted into the perfusate by the liver (Figs. 4 and 5). When livers from Zn-deficient rats were perfused with 60 μM-L-Met, the extracellular concentration of α-ketobutyrate increased with time, reaching a level of 16 nmol/ml after 60 min. This value was some fourfold higher than that seen with the
Zn-supplemented controls (Fig. 4(a)). Increasing the perfusate L-Met to 240 μM resulted in a marked increase in the amount of intracellular and extracellular α-ketobutyrate. Again, the amount of α-ketobutyrate produced by the livers from Zn-deficient rats was greater than that found in the pair-fed Zn-supplemented controls (Fig. 5). Under these conditions the amount of α-ketobutyrate produced by livers from pair-fed rats was not significantly different from that produced by livers from ad lib.-fed rats.

**DISCUSSION**

From the perfusion experiments we found no evidence to suggest that any of the enzymes involved in recycling Hcy back to L-Met (methylaion pathway) were adversely affected by Zn deficiency. Under normal conditions the concentration of Ado-Hcy in the liver is 12–16 nmol/g tissue. This level could not be altered when rats were fed on a Zn-deficient diet for up to 4 weeks (Wallwork & Duerre, 1985). Similarly, the intracellular concentration
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Fig. 5. Concentration of $\alpha$-[carboxyl-$^{14}$C]ketobutyrate in (a) the perfusate and (b) the liver after perfusion of isolated livers with 240 $\mu$M-L-methionine. (●) Liver isolated from ad lib.-fed, (▲) Zn-deficient, and (■) pair-fed rats. Values are means, with their standard errors represented by vertical bars, for four to six separate experiments. *Values within a given line with unlike superscript letters were significantly different (analysis of variance and Scheffe contrasts): $P < 0.05$.

The concentration of this compound could not be altered on perfusion of livers from such animals with L-Met at concentrations up to 240 $\mu$M (Table 2). Therefore, the enzyme S-adenosylhomocysteinase was not affected by Zn deficiency. Under normal conditions the concentration of Ado-Met is dependent on the amount of L-Met available (Hoffman et al. 1980). At low levels of exogenous L-Met, Hcy appeared to be readily recycled to L-Met in livers from all experimental groups. Thus, the intracellular concentration of L-Met and Ado-Met in livers from Zn-deficient rats is maintained within the limits of ad lib.-fed controls. As the extracellular concentration of L-Met increases, the intracellular concentration of both L-Met and Ado-Met increases. Therefore, all the enzymes involved in recycling Hcy to L-Met as well as the methionine adenosyltransferase ($EC\ 2.5.1.6$) appear to be operating normally.

When the results from the perfusion experiments with livers from Zn-deficient rats were compared with that from the pair-fed controls, the concentrations of Ado-Met were lower.
in the pair-fed controls. Diminished L-Met and Ado-Met pools have been observed in livers from pair-fed rats (Wallwork & Duerre, 1985). This was not the result of the impairment of the methionine adenosyltransferase since the level of Ado-Met could be restored to normal concentrations when livers were perfused with large amounts of L-Met (Table 2). The reduced levels of L-Met and Ado-Met are a reflection of the normal response to reduced food intake. The pair-fed rats had periods of up to 36 h during which they had received little food. In contrast to the livers from Zn-deficient animals, the livers from pair-fed rats appeared to respond rapidly to added L-Met. This difference is most likely the result of reduced demand for L-Met in various anabolic reactions in the livers from Zn-deficient rats. Methylation of macromolecules (Wallwork & Duerre, 1985; Wallwork et al. 1986), protein synthesis (Duerre et al. 1977; Fosmire & Sandstead, 1977; Wallwork & Duerre, 1985) and nucleic acid synthesis (Sandstead & Rinaldi, 1969; Williams & Chesters, 1970) are known to be impaired in Zn-deficient animals.

Furthermore, the enzymes involved in the transsulphuration pathway do not appear to be impaired in Zn deficiency. This was evident from the increased rate of catabolism of L-Met, via cystathionine γ-lyase, in livers from Zn-deficient rats. In this pathway cystathionine is broken down irreversibly to α-ketobutyrate and cysteine. When livers from Zn-deficient rats were perfused with 60 μM-L-Met, some 30% of it was metabolized via this enzyme within 1 h. Under these conditions only 5–6% of the L-Met was metabolized via this pathway in the control livers. When the concentration of L-Met in the perfusate was increased to 240 μM, some 60% of it was broken down via the transsulphuration pathway in livers from Zn-deficient rats compared with 40 and 28% for livers from ad lib.- and pair-fed rats respectively. The increased concentration of Hcy and α-ketobutyrate in the perfusate of livers from Zn-deficient rats probably reflects the reduced demand for L-Met in methylation reactions and protein synthesis associated with Zn deficiency. Reports of increased L-Met catabolic products, cysteine (Hsu et al. 1969) and taurine (Hsu & Anthony, 1970) in the urine of Zn-deficient rats are consistent with these observations.

The authors thank Mr Keith C. Leatherbury and Ms Sheryl A. Leland for their technical assistance and Ms LuAnn Johnson for statistical assistance in this study.

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