A novel pathway for the conversion of homocysteine to methionine in eukaryotes

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INTRODUCTION

Three closely related sulphur-containing amino acids, namely methionine, cysteine and homocysteine, are found in all cells. Two of these, methionine and cysteine, are activated and attached to the cognate tRNA by the corresponding aminoacyl-tRNA synthetases. Activation was studied by measuring the incorporation of radioactive amino acid into unlabelled trichloroacetic-acid insoluble materials in the absence of protein synthesis. Homocysteine synthetase activity was found in the crude extract and in the purified multi-enzyme complex of aminoacyl-tRNA synthetases. On a molar basis, the activation of methionine by the crude extract was five times higher than the activation of homocysteine. There was a partial loss of Hcy-tRNA synthetase activity with the enzyme by the crude extract was five.

Similar to several other synthetases, Met-tRNA synthetase also possesses proofreading activity [2]. When bacterial Met-tRNA synthetase was incubated with homocysteine, the enzyme misactivated this amino acid, producing the corresponding aminoacyl-AMP intermediate. The misactivated aminoacyl-AMP intermediate that remained attached to the enzyme was hydrolysed, either immediately or during the transfer of the amino acid to the enzyme-bound tRNA. Hydrolysis of homocysteinyl-AMP gave rise to homocysteine thiolactone and AMP [2]. Based on this and other experiments in vitro, it was postulated that the formation of large quantities of homocysteine thiolactone by several types of malignant cells is due to the proofreading activity of Met-tRNA synthetase [3]. Misactivation was measured by measuring the thiolactone formed.

For most aminoacyl-tRNA synthetases, the extent of misactivation in systems in vitro varies with the assay conditions [4]. If one assumes that the proofreading activity of Met-tRNA synthetase alone is responsible for homocysteine thiolactone formation, the extent of homocysteine misactivation reported is extremely high both in vivo and in vitro [2,3,5]. One would, therefore, expect a small fraction of the misactivated homocysteine to escape the proofreading activity of the synthetase and appear as Hcy-tRNA.

We investigated this activation/misactivation reaction from a different angle. Instead of measuring the formation of homocysteine thiolactone, we measured the incorporation of [¹⁴C]homocysteine into tRNA. Preliminary experiments were conducted using rabbit reticulocyte lysate. Later a purified aminoacyl-tRNA synthetase complex isolated from rabbit liver was used. Our data indicate that homocysteine activation is real and tRNA-bound homocysteine is converted to methionine through a novel methylation reaction, and that homocysteine may, in fact, be the preferred amino acid for aminoacylation of initiator tRNA in eukaryotes.

We propose that homocysteine thiolactone is formed under conditions when there is an inadequate methylation of Hcy-tRNA to Met-tRNA.

MATERIALS AND METHODS

Chemicals

N-Hydroxysuccinimide-activated Sepharose and Sepharose CL 6B were obtained from Pharmacia Biotech AB, Uppsala, Sweden. Benzoylated naphthoylated DEAE (BND)-cellulose was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of the purest grade available from standard suppliers.

Abbreviations used: BND, benzoylated naphthoylated DEAE; DTT, dithiothreitol; PEG, poly(ethylene glycol); TCA, trichloroacetic acid.

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Isotopes

$[^{35}S]$Methionine (SJ 235) was obtained from Amersham International plc, Little Chalfont, Bucks, U.K. $[^{14}C]$Homocysteine thiolactone (specific activity, 56 mCi/mmole) was custom synthesized (Amersham).

Isolation of tRNA from sheep liver

Fresh sheep liver, obtained from the slaughter house, was chilled in ice and used immediately. The isolation of total RNA was carried out essentially according to Chomczynski and Sacchi [6]. The tRNA was separated from total RNA by the method described by Fournier et al. [7] with slight modifications. The step involving adsorption and elution from DEAE-cellulose was performed twice. More than 90% of purified RNA eluted at the expected position for tRNA from an FPLC Suparose 12 column.

Partial purification of Met-tRNA, from total tRNA

The procedure described by Fournier et al. [7] was followed. Total tRNA (40 mg) was separated on a BND-cellulose column (40 x 2.5 cm). tRNA was eluted with a 500 ml linear gradient of 0.4–1 M NaCl in 10 mM MgSO$_4$ [8]. Fifty 10 ml fractions were collected. This method does not yield baseline separation of tRNA species but enrichment of certain tRNA species can be achieved. The first 8 fractions, which had both methionine- and homocysteine-acceptance activity were pooled (BND-cellulose fraction 1). BND-cellulose fraction 2 comprised fractions 36–50, which had methionine-acceptance activity. Fractions 9–35 had poor activity for either amino acid, and were not used. The tRNA fractions were diluted to reduce the NaCl concentration before ethanol precipitation.

Decacylation of tRNA

The purified tRNA was decacylated by incubation in 0.1 M ammonia/HCl buffer, pH 9.5, for 30 min. The uncharged tRNA was precipitated with ethanol at pH 5.0, and stored in TE buffer (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5).

Preparation of an affinity column containing covalently bound tRNA

A total of 500 units of tRNA (monitored at A$_{260}$), purified as described above, was coupled to an $N$-hydroxysuccinimide-activated Sepharose column (5 ml), as recommended by the manufacturer (Pharmacia Biotech). The coupling efficiency of tRNA to the column was 85%.

Preparation of $[^{14}C]$homocysteine from $[^{14}C]$homocysteine thiolactone

For stability, custom synthesized $[^{14}C]$homocysteine was supplied as homocysteine thiolactone in dilute HCl. This thiolactone was converted to homocysteine by hydrolysis at alkaline pH. One vol. of the stock solution of homocysteine thiolactone was mixed at room temperature with 3 vol. of 0.5 M ammonia/HCl buffer, pH 9.5, and 1 vol. of 25 mM dithiothreitol (DTT). After 15 min at 20°C, this mixture was used directly in the homocysteine synthetase assay.

Separation and characterization of sulphur containing amino acids

Two separate methods were used to characterize the radioactive amino acids released from tRNA aminoc酰ylated with $[^{14}C]$homocysteine. In the first method, HPLC separation of the released amino acids was performed using two independent systems in order to differentiate between homocysteine thiolactone and methionine. The first system was developed for the identification of thiols, including the oxidized species [9]. This system does not separate methionine from homocysteine thiolactone. The second HPLC was performed using a system developed for the identification of amino acids after derivatization with o-phthalaldehyde [10]. In the second method, separation and identification were achieved by TLC as described previously [11].

Assay of aminoacyl-tRNA synthetase activity

Met-tRNA synthetase, and Hcy-tRNA synthetase activities were measured at 30°C. The standard assay mixture, contained in 50 µl final volume, was 50 mM Tris/HCl (pH 7.4)/0.1 mM EDTA/1 mM DTT/5 mM magnesium acetate/100 mM KCl/2 mM ATP/0.5 mM CTP containing 2 units of tRNA (monitored at A$_{260}$), 1 µCi of 100 µM $[^{35}S]$methionine or 0.25 µCi of 100 µM $[^{14}C]$homocysteine and an appropriate quantity of enzyme (ranging from 0.5–100 µg of protein). The samples were incubated for 10 min and 40 µl of each sample was then applied to Whatman 3-mm filter paper disks, which had been soaked in 10% (w/v) trichloroacetic acid (TCA) containing 100 mM mercaptopethanol and 1 mM methionine or 1 mM homocysteine. The presence of mercaptoethanol during TCA precipitation reduced the background activity in the homocysteine samples. After 15 min, the samples were washed 7 times with 5% (w/v) TCA and twice with ethanol. The radioactivity on the dried filter paper was measured by scintillation spectroscopy. The reaction mixture was increased to 400 µl in experiments where labelled tRNA was extracted with phenol after incubation. One unit of enzyme activity was defined as the amount of enzyme required to catalyse the formation of 1 nmol of aminoacyl-tRNA in 10 min at 30°C.

Purification of the multi-enzyme complex of aminoacyl-tRNA synthetases

Fresh rabbit liver was transported from the animal house to the laboratory in ice-cold buffer and used immediately for isolation of the enzyme. The purification procedure was similar to that of Kellermann et al. [12]. All steps of the purification procedure were carried out at 4°C. Unless otherwise stated, all buffers contained 1 mM DTT, 10% (v/v) glycerol and 0.1 mM PMSF. Fresh rabbit liver (50 g) was homogenized in 100 ml of buffer A [50 mM Tris/HCl, pH 7.5/5 mM magnesium acetate/10% (v/v) glycerol/1 mM DTT/0.1 mM PMSF], for 1 min in a food processor and then for 2 min in a tissue homogenizer. A stock solution of 50% (w/w) poly(ethylene glycol) (PEG) (in 50 mM Tris/HCl, pH 7.4/5 mM MgCl$_2$/1 mM DTT) was slowly added to the homogenate, with stirring, to a final concentration of 2% (w/w) PEG. The mixture was stirred for 30 min and centrifuged at 13000 g for 10 min at 4°C. The supernatant was collected and precipitated once more with PEG to a final concentration of 4% (w/w) and centrifuged as described above. Most of the enzyme activity in the clear supernatant was precipitated when the PEG concentration was increased to 9% (w/w). This precipitate was collected and dissolved in 10 ml of buffer B [25 mM potassium phosphate, pH 7.5/5 mM magnesium acetate/10% (v/v) glycerol/1 mM DTT/0.1 mM PMSF] and centrifuged at 13000 g for 10 min at 4°C. The supernatant was passed through a 0.8-µm Millipore filter and the filtered protein solution (2 ml) was applied to a XK 26/70 column (Pharmacia) containing Sepharose 4B CL equilibrated with buffer B. The void volume of the column was 110 ml and the total volume was 325 ml. The proteins were eluted with buffer B at a flow rate of 40 ml/h.
Seventy 5-ml fractions were collected and assayed for Met- and Hcy-tRNA synthetase activities. The final purification step was achieved using affinity chromatography according to the method of Kellermann et al. [12]. A Sepharose affinity column containing covalently bound tRNA was equilibrated with buffer B. Fractions containing synthetase activity were pooled and passed twice through this column at a flow rate of 20 ml per h. The column was then washed with 5 ml of buffer B. The bound enzyme was eluted with 20 ml of buffer B containing 200 mM potassium phosphate. Fractions (0.5 ml) were collected and assayed for synthetase activity.

**RESULTS**

**Homocysteine incorporation into tRNA by rabbit crude liver extract**

Measurement of the aminoaacylation reaction in crude extracts is difficult and unreliable. We therefore measured this reaction in the supernatant obtained after the first PEG precipitation of the crude extract [12]. Endogenous free amino acids were removed by passing the extract through a Sephadex G-50 column. Before the addition of radioactive amino acid, the reaction mixture was preincubated for 5 min with 10 µM puromycin to block any residual protein synthesis. Table 1 shows the Met- and Hcy-tRNA synthetase activities in the presence and absence of the competing amino acid. To our surprise, addition of non-radioactive methionine did not reduce the incorporation of homocysteine into tRNA. Furthermore, the Met-tRNA synthetase activity was not reduced in the presence of homocysteine. The fact that homocysteine incorporation was almost completely abolished in the absence of ATP proved that the observed incorporation was real and was not due to contamination by free homocysteine. On a molar basis, the incorporation of methionine into tRNA was 4–5-fold higher than that of homocysteine (Table 1).

**Hcy- and Met-tRNA synthetase activities of the multi-enzyme complex of aminoacyl-tRNA synthetases**

Mammalian Met-tRNA synthetase is associated with several other synthetases in a multi-enzyme complex [13]. This complex is fairly easy to isolate by PEG fractionation followed by gel filtration on a Sepharose C column. Figure 1 shows the elution profile of Met- and Hcy-tRNA synthetase activities in the eluate from the Sepharose column. The two synthetase activities almost overlapped and both revealed a heterogeneity in terms of apparent molecular mass. These multi-enzyme complex fractions were pooled and purified further by affinity chromatography on a Sepharose column containing covalently-bound tRNA. The elution profile from the affinity column is shown in Figure 2. There was a gradual loss of Hcy-tRNA synthetase activity during purification procedure (Table 2). When the multi-enzyme synthetase complex was assayed in the presence of late-eluting protein fractions from the Sepharose C column, some of the lost activity was regained suggesting the requirement of an additional factor for the maintenance of Hcy-tRNA synthetase activity. The specific activity of Hcy-tRNA synthetase in the multi-enzyme complex was low compared with that of methionine. As in the crude extract, the Hcy-tRNA synthetase activity of the multi-enzyme complex was not decreased when the assay was conducted in the presence of methionine.

**Identification of the amino acid released from labelled tRNA after alkaline hydrolysis**

The conditions used in the present aminoaacylation reaction (low concentration of Mg²⁺ and the presence of spermidine) are known to increase translational fidelity in vitro [14]. In order to confirm that homocysteine is covalently bound to tRNA as an aminoacyl derivative, tRNA was isolated from the reaction mixture as described previously [6] and residual free amino acids were removed by five consecutive ethanol precipitations. Amino acids attached to tRNA as an aminoacyl ester were hydrolysed by incubating under mild alkaline conditions. Ninety % of the radioactivity was released from tRNA as free amino acid during this step. The amino acids released from tRNA labelled with

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**Table 1** Aminoacyl-tRNA synthetase activity in rabbit liver extracts

<table>
<thead>
<tr>
<th>Concentration of unlabelled amino acid</th>
<th>Aminoaacyl-tRNA synthetase activity (pmol amino acid incorporated/10 min)</th>
<th>Homocysteine</th>
<th>Methionine</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.4 ± 0.5</td>
<td>12.5 ± 1.2</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>100 µM Methionine</td>
<td>2.5 ± 0.6</td>
<td>–</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>100 µM Methionine–ATP</td>
<td>0.4 ± 0.1</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>100 µM Homocysteine</td>
<td>–</td>
<td>12.6 ± 1.1</td>
<td>5.2</td>
<td></td>
</tr>
</tbody>
</table>
Active fractions from the Sepharose C column were pooled and passed twice through the affinity column equilibrated with buffer B. The column was washed with 5 ml of buffer B before elution with 20 ml of buffer B containing 200 mM of potassium phosphate. Fractions (0.5 ml) were collected, and 2 µl or 4 µl from each fraction was used for estimation of Met-tRNA synthetase or Hcy-tRNA synthetase activity respectively.

### Table 2 Loss of Hcy-tRNA synthetase activity on purification

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Homocysteine</th>
<th>Methionine</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>0.06</td>
<td>0.31</td>
<td>5.2</td>
</tr>
<tr>
<td>4–9% (w/v) PEG precipitate</td>
<td>0.51</td>
<td>2.95</td>
<td>5.8</td>
</tr>
<tr>
<td>Sepharose fraction 1</td>
<td>1.20</td>
<td>11.64</td>
<td>9.7</td>
</tr>
<tr>
<td>Sepharose fraction 2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sepharose fraction 1 + 2</td>
<td>1.43</td>
<td>11.62</td>
<td>8.1</td>
</tr>
<tr>
<td>Affinity-column fraction</td>
<td>11.00</td>
<td>118.80</td>
<td>10.8</td>
</tr>
</tbody>
</table>

[^14]C homocysteine were identified both by HPLC [9,10] and TLC [11]. The distribution of radioactivity in the various fractions from two HPLC runs is shown in Figure 3. Table 3 shows the distribution of radioactivity recovered from thin layer chromatograms. When the homocysteine charging reaction was carried out with purified multi-enzyme complex, more than half of the radioactivity applied on the thin layer plate was recovered as homocysteine plus homocystine. Around 20% of radioactivity was recovered as homocysteine thiolactone (Table 3). When the same charging reaction was carried out with crude extract, more than half of the applied radioactivity was recovered as methionine. Radioactivity recovered as homocysteine plus homocystine was around 17%. These results suggest the existence of a hitherto unknown methylation pathway for the conversion of homocysteine to methionine.

### Characterization of homocysteine-accepting tRNA

Conversion of tRNA-bound homocysteine to methionine (Table 3) suggested that homocysteine was attached to Met-tRNA. Since there are two species of methionine accepting tRNAs, namely initiator tRNA (Met-tRNA^i^) and elongator tRNA (Met-tRNA^m^), we measured the homocysteine accepting activity of partially purified initiator tRNA. In Table 4, the homocysteine
Homeocysteine and homocystine in this solvent system were 0.52 ± 0.08 µM and depleted for Met-tRNAi (BND–cellulose fraction 2) were obtained as described in the Materials and methods section.

The precipitate (7 M NaCl) was dissolved in 0.1 M sodium acetate buffer, pH 5, and re-precipitated five times. Amino acid bound to tRNA was deacylated as described in the Materials and methods section. The amino acids in the supernatant were concentrated in a stream of N2 and subjected to TLC [11]. Radioactivity recovered in this case is the sum of homocysteine and homocystine values.

The charging reaction was conducted with 16 A260 units of purified tRNA in a final volume of 400 µl. After 15 min at 30 °C, RNA was extracted with phenol and precipitated with ethanol. The precipitated RNA was dissolved in 0.1 M sodium acetate buffer, pH 5, and re-precipitated five times. Amino acid bound to tRNA was deacylated as described in the Materials and methods section. The RNA was removed by ethanol precipitation. The amino acids in the supernatant were concentrated in a stream of N2 and subjected to TLC [11]. Radioactivity recovered in this case is the sum of homocysteine and homocystine values.

A novel pathway for the conversion of homocysteine to methionine was found [16]. Very little is known about the function of the remaining 30 kDa polypeptide removed from the 108 kDa Met-tRNA synthetase of the multi-enzyme complex. The multi-enzyme synthetase complex from rabbit liver contains ten large polypeptides and some additional low-molecular-mass polypeptides [15]. Met-tRNA synthetase activity is present in a 108 kDa polypeptide [15]. Mild proteolysis of this complex releases a truncated form (78 kDa) of Met-tRNA synthetase which is catalytically active [16].

The wary interaction of homeocysteine with the acceptor site of tRNA is reduced significantly in the presence of the homocysteine synthetase complex (Table 2) indicates that the two activities are not completely identical. These results, however, do not rule out the presence of a common component in both enzymes. The data presented in Table 2 indicate that homeocysteine activation requires an additional factor. The multi-enzyme synthetase complex from rabbit liver contains ten large polypeptides and some additional low-molecular-mass polypeptides [15]. Met-tRNA synthetase activity is present in a 108 kDa polypeptide [15]. Mild proteolysis of this complex releases a truncated form (78 kDa) of Met-tRNA synthetase which is catalytically active [16]. Very little is known about the function of the remaining 30 kDa polypeptide removed from the 108 kDa Met-tRNA synthetase of the multi-enzyme complex.

Conversion of tRNA-bound [14C]homeocysteine to methionine was significant in the crude extract (Table 3). At the beginning of this study, we reasoned that radioactive contamination of our [14C]homeocysteine thiolactone with methionine could be responsible for the apparent homeocysteine activation and its appearance in tRNA as methionine. Therefore we checked the radiochemical purity of our labelled homeocysteine thiolactone and found that it was greater than 95%. A second possible scenario is that the conversion of homeocysteine to methionine could have occurred at the level of free amino acid. The time taken for the aminoacylation reaction was short (10 min). If significant conversion of homeocysteine to methionine occurred during this short period, one would have expected that nonradioactive methionine would inhibit the incorporation of homeocysteine into tRNA. As shown in Table 1, the incorporation of homeocysteine into tRNA was insensitive to the presence of nonradioactive methionine and vice versa. Ability to accept homeocysteine was present in the tRNA fraction enriched for initiator tRNA. The ability of this tRNA fraction to accept methionine was lower than its ability to accept homeocysteine (Table 4).

Amino acid incorporated/assay (pmol) was measured using the 4–9% PEG centrifugation method [17]. Bacterial Met-tRNA synthetase does not discriminate between bacterial Met-tRNA and Met-tRNA, but it does discriminate between eukaryotic Met-tRNA and Met-tRNA, and can attach methionine to mammalian Met-tRNA, but not to Met-tRNA [18].

**Table 3** Identification of amino acids released from radioactively labelled tRNA charged in vitro

<table>
<thead>
<tr>
<th>Homocysteine + homocystine</th>
<th>Homocysteine thiolactone</th>
<th>Methionine</th>
<th>Total recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>670</td>
<td>234</td>
<td>22</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>685</td>
<td>247</td>
<td>23</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>206</td>
<td>54</td>
<td>23</td>
</tr>
</tbody>
</table>

**Table 4** Homeocysteine accepting activity of different tRNA samples

Incorporation of homeocysteine and methionine into tRNA was measured using the 4–9% PEG precipitation (7 µg protein). The tRNA fractions enriched for Met-tRNAi (BND–cellulose fraction 1) and depleted for Met-tRNA (BND–cellulose fraction 2) were obtained as described in the Materials and methods section.

<table>
<thead>
<tr>
<th>IRNA fraction</th>
<th>Amino acid incorporated/assay (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated IRNA</td>
<td></td>
</tr>
<tr>
<td>BND–cellulose fraction 1</td>
<td>5.9</td>
</tr>
<tr>
<td>BND–cellulose fraction 2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Since homeocysteine is a non-protein amino acid, it is natural to consider the attachment of this amino acid to tRNA as a misactivation reaction by Met-tRNA synthetase. Misactivation of an amino acid is reduced significantly in the presence of the cognate amino acid. We found however, that the homeocysteine activation is unaffected by the presence of equimolar concentration of methionine (Table 1). The inability of non-radioactive homocysteine to influence the incorporation of methionine (Table 1) also suggests that these two amino acids are not competing for the same enzyme and/or the same tRNA species. The concentration of methionine and homocysteine used in the experiments was 100 µM and it is possible that this concentration of homocysteine is high compared with the concentration in vivo, but it is far below the concentration used in previous experiments in vivo [2].

Loss of Hcy-tRNA synthetase activity upon purification of the multi-enzyme synthetase complex (Table 2) indicates that the activity is unaffected by the presence of equimolar concentration of methionine (Table 1). The inability of non-radioactive methionine to accept homocysteine further indicates that homocysteine is activated and attached only to Met-tRNAi.
Methionine attached to bacterial initiator tRNA by the bacterial enzyme is modified by the formylating factor. Homocysteine-activating enzyme resembles the bacterial Met-tRNA synthetase in two ways. Both enzymes acylate only mammalian initiator tRNA and both enzymes modify the acylated initiator tRNA. Thus the aminoacylation of initiator tRNA with homocysteine and its subsequent methylation to methionine is quite analogous to the aminoacylation of bacterial initiator tRNA where methionine is converted to formyl methionine-tRNA. Our data suggests that the methylation reaction is essential and we propose that, in the absence of methylation, Hcy-tRNA is hydrolysed to produce homocysteine thiolactone. Notably, previous studies have shown that homocysteine thiolactone formation is especially high in cells defective in methionine metabolism [3].

In conclusion, our results show that homocysteine is activated and attached to initiator tRNA and methylated to Met-tRNA by a methylating factor. This explains why activated homocysteine is not found in proteins. Since N-terminal methionine is released from the polypeptide after chain initiation, homocysteine converted by this pathway can enter the cellular methionine pool. In our assay system, the aminoacylation of partially purified initiator tRNA by homocysteine was more efficient than its aminoacylation by methionine (Table 4). The existence of two separate pathways for the formation of Met-tRNA, and a coupling of one pathway to a novel methyl-transfer reaction, most likely have important regulatory significance.

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REFERENCES


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